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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/489,850	01/24/2000	Diane Van Alstyne	51916/107	6341

7590 12/18/2006

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EXAMINER	
DUFFY, PATRICIA ANN	
ART UNIT	PAPER NUMBER
1645	

SHORTENED STATUTORY PERIOD OF RESPONSE	MAIL DATE	DELIVERY MODE
3 MONTHS	12/18/2006	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

If NO period for reply is specified above, the maximum statutory period will apply and will expire 6 MONTHS from the mailing date of this communication.

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Office Action Summary	Application No.	Applicant(s)	
	09/489,850	ALSTYNE ET AL.	
	Examiner	Art Unit	
	Patricia A. Duffy	1645	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *02 March 2006*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *14-25* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *14-25* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

The response filed 3-2-06 has been entered into the record.

Specification

A substitute specification including the text and changes of the preliminary amendment filed 1-24-00 is required pursuant to 37 CFR 1.125(a) because: the preliminary amendment filed 1-24-00 could not be entered in multiple instances because they requested line numbers did not contain the recited textual material.

A substitute specification must not contain new matter. The substitute specification must be submitted with markings showing all the changes relative to the immediate prior version of the specification of record. The text of any added subject matter must be shown by underlining the added text. The text of any deleted matter must be shown by strike-through except that double brackets placed before and after the deleted characters may be used to show deletion of five or fewer consecutive characters. The text of any deleted subject matter must be shown by being placed within double brackets if strike-through cannot be easily perceived. An accompanying clean version (without markings) and a statement that the substitute specification contains no new matter must also be supplied. Numbering the paragraphs of the specification of record is not considered a change that must be shown.

Priority

It is noted that this application appears to claim subject matter disclosed in three prior Applications. The current status of all non-provisional parent applications referenced should be updated.

Drawings

The proposed drawings corrections in this application have been accepted. ***The corrected drawings are required in reply to the Office action to avoid abandonment of the application. The requirement for corrected drawings will not be held in abeyance.***

Specification

The title of the invention is not descriptive of the now claimed invention. A new title is required that is clearly indicative of the invention to which the claims are directed.

Oath/Declaration

The oath or declaration is defective. A new oath or declaration in compliance with 37 CFR 1.67(a) identifying this application by application number and filing date is required. See MPEP §§ 602.01 and 602.02.

The oath or declaration filed 3-2-06 is defective because it is not executed by inventor Diane Van Alstyne.

Information Disclosure Statement

No information disclosure statement has been filed in this application.

Election/Restrictions

Applicant's election of Group II, Specie F, SEQ ID NO:20 in the response of 3-2-06 is acknowledged. ***Upon reconsideration, the restriction requirement between Groups I and II is withdrawn. The specie election is maintained.*** Because applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.03(a)).

Claim Objections

Claims 16 is objected to because of the following informalities: the independent claim recites the acronym "MRHAS" that is not first defined in the independent claim. While acronyms are permitted in the claims, they must be fully defined in any independent claim. Appropriate correction is required.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 14-25 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

As to claims 14, 15, 18, 19, 20, 22 and 23, the claims are drawn to a protective effect *in vivo* against challenge to comprising administering an "effective amount" of a composition comprising a monoclonal antibody or binding fragment thereof that binds a meningitis related homologous antigenic sequence (MRHAS) shared by viral and/or

bacterial meningitis etiological agents. The claims are interpreted as prevention of infection as protection indicates prevention of infection or disease (see specification page 76, lines 15-22).

As to claims 16, 17, 21, 24 and 25, the claims are drawn to treating a patient infected with a meningitis etiological virus and/or bacteria to significantly clear said virus and/or bacteria comprising administering a therapeutically effective amount of a composition comprising a monoclonal antibody or binding fragment thereof that binds a meningitis related homologous antigenic sequence (MRHAS) shared by viral and/or bacterial meningitis etiological agents. Treatment encompasses curing or easing symptoms (see MSN Encarta Dictionary).

The sole teaching of the specification are drawn to an assessment of the ability of the monoclonal antibody SP8, produced by the cell line 11E-1 to reduce bacteremia in a challenge model and also a study of the survival rate (see pages 74-78, Example 4). In the instant case the monoclonal antibody binds the sequence QQPPE in *S. pneumoniae* and the animals were challenged with *H. influenzae* that has the homologous sequence QVQNNKP. The antibody was first provided to the animal and then after 24 hours the animals were challenged with the *H. influenzae*. Table 10 indicates that while the monoclonal antibody reduced the number of bacteria in the blood when administered prior to exposure, so did the negative control and the positive control. The specification teaches single RV1 monoclonal antibody that defines family of homologous cross-reacting septapeptide antigens in viruses and bacteria known to cause meningitis (see pages 31-33). The specification lacks any description of any *in vitro* assay of biological activity for the monoclonal antibody RV1. The specification does not teach that clearing etiological agents provide for "curing or easing symptoms" as clearly encompassed by the term "treating" and does not teach protection from in vivo challenge for the following reasons.

At the time that the invention was made, none of the disclosed antigens were known or demonstrated by the specification to provide active immunity or protective antibodies

thereto to provide passive immunity against the cognate disease or disorder, much less a heterologous disease or disorder for which the antibody is cross-reactive. The ability of an antigen to bind an antibody or the ability of the antibody to recognize its cognate antigen is not recognized by the art to demonstrate therapeutic efficacy of a vaccine or antibody. Many references teach that the presence of antibody or ability to generate an antibody does not correlate with efficacy. Applicants are essentially claiming passive immunization as opposed to active immunization and as such, the antibody must have the claimed properties. There is absolutely no demonstration of protective immunity upon administration in any animal model of disease. The art is replete with evidence that the ability to produce an antibody (immunogenicity) is insufficient to correlate with protection from infection. See for example Feng et al (Infection and Immunity, 64(1):363-365, 1996) that teaches that P55, is an immunogenic but nonprotective 55-kilodalton *Borrelia burdorferi* protein in murine lyme disease. It is well recognized in the vaccine art, that it is unclear whether an antigen(s) derived from a pathogen will elicit protective immunity. Ellis, R.W. (Chapter 29 of "VACCINES" [Plotkin, S.A. et al. (eds) published by W. B. Saunders company (Philadelphia) in 1988, especially page 571, 2nd full paragraph] exemplifies this problem in the recitation that "The key to the problem (of vaccine development) is the identification of that protein component of a virus or microbial pathogen that itself can elicit the production of protective antibodies.... and thus protect the host against attack by the pathogen". The specification fails to teach even one monoclonal antibody alone or in combination with other antibodies does in fact confer protection from infection (*in vivo* challenge) or have some recognized therapeutic efficacy (treatment), as is required by the claimed invention. The art in 2001 clearly recognized that the ability to produce an antibody does not correlate with protection from infection (Chandrashekar et al US Patent NO. 6,248,329 column 1, lines 35-40). Therefore, the fact that an antibody binds the antigen, does not provide any indication of the usefulness of the antibody in protection from infection. As such, one skilled in the art would have

ample reasons to doubt the ability to use a composition comprising the monoclonal RV1 antibody as a passively immunizing vaccine. Similarly, there is no evidence that any of the antibodies provide for treatment of infection post exposure by any bacteria or virus. For rubella in particular, the art recognizes no efficacy of post-exposure passive immunization (see Public Health Agency of Canada: Vaccine-Preventable Diseases Rubella, page 12, passive immunization).

Applicants characterize that in severe meningoccoal infections bacteremia, petechiae and shock may develop (see page 2, lines 15-20). The skilled artisan also readily recognizes that blood is a sterile body fluid. Bacteria present in the blood provide for a disease state called bacteremia. The fact that all the animals in Example 4 bacteria in the blood, indicates that the monoclonal antibody cannot protect from challenge *in vivo* and that all the animals progressed from a peritoneal infection to a blood infection. So, the antibody even when administered 24 hours in advance the monoclonal antibody cannot protect. Further, the specification speculates that the protective effect may block the common MRHAS-mediated entry of the meningitis organisms into carrier monocytes (see specification page 17, lines 30-38). The specification does not teach protection from infection because all of the animals with all antibodies clearly have bacteria in blood 24 hours after inoculation into the peritoneum. Therefore, it is clear that the bacteria survived and moved from the peritoneum into the blood and infection is not prevented or protection from challenge not achieved. There is no demonstration of any *in vitro* activity of any other anti-bacterial monoclonal antibody that is correlative or predictive of protection *in vivo* against any other disclosed bacterial antigen as claimed. There is no demonstration of any *in vitro* activity that provides for in vivo efficacy (cure or alleviation of symptoms or protection from infection) with respect to treatment of any patient infected with a meningitis etiological agent as claimed. There is no evidence of clearance of the agent provides for treatment as claimed. Treatment is conventionally defied as a procedure, or technique for curing or alleviating a disease, injury, or condition. There is no

evidence that any monoclonal antibody, even those that promote clearance of bacteria or viruses alleviate the disease or condition of the patient for the reasons set forth below. Additionally, the specification fails to disclose that the RV1 or SP8 monoclonal antibody provides for protection against any viral meningitis agents using *in vitro* or *in vivo* assays that correlate with protection from *in vivo* challenge. In particular, the specification contemplates protection from HIV using the RV1 monoclonal antibody. It has been well known in the art that retroviral infections in general, and HIV infections in particular, are refractory to anti-viral therapies. The obstacles to therapy of HIV are well documented in the literature. These obstacles include: 1) the extensive genomic diversity and mutation rate associated with the HIV retrovirus, particularly with respect to the gene encoding the envelope protein; 2) the fact that the modes of viral transmission include both virus-infected mononuclear cells, which pass the infecting virus to other cells in a covert manner, as well as via free virus transmission; 3) the existence of a latent form of the virus; 4) the ability of the virus to evade immune responses in the central nervous system due to the blood-brain barrier; and 5) the complexity and variation of the pathology of HIV infection in different individuals. The existence of these obstacles establish that the contemporary knowledge in the art would not allow one skilled in the art to use the claimed invention with a reasonable expectation of success and without undue experimentation. Further, it has been well known in the art that individuals infected with HIV produce neutralizing antibodies to the virus, yet these antibodies are not protective and do not prevent the infection from progressing to its lethal conclusion. Further, as taught by Fahey et al. (Clin. Exp. Immunol. 88: 1-5, 1991) , clinical trials using a variety of immunologically based therapies have not yielded successful results in the treatment and/or prevention of HIV infection (see Table 1). Fahey et al. also disclose in vitro-in vivo discrepancy involved in applying Receptor-Directed Treatments involving CD4-specific inhibitors (see page 3, column 1). Fahey et al. discloses that monoclonal antibody therapies have not provided any clinical benefits and "it is not clear how adding these

additional antibodies would make a difference" (see page 3, second column, third full paragraph). In support, Daar et al. (PNAS 87: 6574-6578, 1990) discloses high concentrations of soluble CD4 required for neutralizing infection poses a formidable problem for such treatment of HIV-1 infection in vivo (see entire document including Abstract and Discussion). Haynes et al. (Science 271: 324-328, 1996) also teaches the limitations of protective immunity to HIV infection, including that "Current animal models of either HIV or simian immunodeficiency virus (SIV) fall short of precisely mirroring human HIV infection" and that "lacking these models, researchers must turn towards human clinical trials to answer many of the difficult questions about HIV pathogenesis and HIV vaccine development" (see page 40, column one, third paragraph). Fox (Biotechnology 12: 128, 1994) also discloses that ability to treat and/or prevent HIV infection is highly unpredictable and has met with very little success. Sommerfelt et al. (J. Gen. Virol. 76: 1345-1352, 1995) discloses that certain antibodies directed against CD18, CD11b and CD11c inhibited HIV-1 induced syncytium formation but not entry (Abstract). Also, certain anti-ICAM-3 antibodies inhibited HIV-1 specific entry but not syncytium formation and only one antibody inhibited HIV-1 induced syncytium formation , entry and infectivity under in vitro conditions (Abstract and Results). Here, it is noted that inhibition was not complete under in vitro conditions using cell lines. Also, Sommerfelt et al. disclose that the inhibitory anti-adhesion antibodies varied on the cell type tested as well as the type of assay (see Results and Discussion). In view of the lack of predictability of the art to which the invention pertains the lack of established clinical protocols for effective methods to suppress the infection of leukocytes with HIV wherein said method comprises administering to a subject exposed to or infected by HIV, including the use of adhesion-based reagents, undue experimentation would be required to practice the claimed methods with a reasonable expectation of success, absent a specific and detailed description in applicant's specification of how to effectively practice the claimed

methods and absent working examples providing evidence which is reasonably predictive that the claimed methods are effective for suppressing HIV infection *in vivo*.

The specification fails to provide any written description of *in vivo* activity for a monoclonal antibody (RV1 or SP8) or any other monoclonal antibody as claimed that is protective for in vivo challenge, curative or alleviates symptoms of disease or disorder as claimed.

In view of the state of the art with respect to use of monoclonal antibodies for therapeutic in 1993, the unpredictability of the art as it relates to correlating antigenicity with protection from infection and the lack of either *in vitro* assays that correlate with *in vivo* efficacy or *in vivo* models that correlate with efficacy for treatment, it would require undue experimentation on the part of the skilled artisan to use the monoclonal antibodies for *in vivo* therapeutics (cure or alleviate) and protection from infection as claimed.

Claim 14-25 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

16, 17, 21, 24 and 25, are indefinite in the use of the term "effective amount" because it is unclear what amount is effective for (i.e. the result variable). Further, the specification does not define or determine or teach an "effective amount". Effective amounts of monoclonal antibodies were not established in the art at the time that this invention was made. Monoclonal antibodies were not in routine clinical use at the time that this invention was made. As such, the skilled artisan would be unable to determine the metes and bounds of the "effective amount".

As to claims 16, 17, 21, 24 and 25, the claims are prima facie indefinite in the use of the term "therapeutically effective amount" because the therapeutic outcome is not defined in the specification or claimed. The specification does not teach what symptoms are treated. The art at the time in 1993 does not teach therapeutically effective amounts

of monoclonal antibodies. Monoclonal antibodies were not in routine clinical use at the time that this invention was made. As such, the skilled artisan would be unable to determine the metes and bounds of the "effective amount".

Status of the Claims

All claims stand rejected.

Conclusion

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Patricia A. Duffy whose telephone number is 571-272-0855. The examiner can normally be reached on M-Th 6:30 am - 6:00 pm. If attempts to reach the examiner by telephone are unsuccessful, the examiner's Supervisory Examiner Jeffrey Siew can be reached on 571-272-0787.

The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patricia A. Duffy, Ph.D.
Primary Examiner
Art Unit 1645

Notice of References Cited	Application/Control No. 09/489,850	Applicant(s)/Patent Under Reexamination ALSTYNE ET AL.	
	Examiner Patricia A. Duffy	Art Unit 1645	Page 1 of 2

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,248,329	06-2001	Chandrashekar et al.	424/191.1
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
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FOREIGN PATENT DOCUMENTS

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	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Feng et al (Infection and Immunity, 64(1):363-365, 1996) ✓
	V	Ellis, R.W. (Chapter 29 of "VACCINES" [Plotkin, S.A. et al. (eds) published by W. B. Saunders company (Philadelphia) in 1988, especially page 571, 2nd full paragraph] ✓
	W	Fahey et al. (Clin. Exp. Immunol. 88: 1-5, 1991) ✓
	X	Daar et al. (PNAS 87: 6574-6578, 1990) ✓

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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	Examiner Patricia A. Duffy	Art Unit 1645	Page 2 of 2

U.S. PATENT DOCUMENTS

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	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Haynes et al. (Science 271: 324-328, 1996)
	V	Fox (Biotechnology 12: 128, 1994)
	W	Sommerfelt et al. (J. Gen. Virol. 76: 1345-1352, 1995)
	X	MSN Encarta Dictionary; definition of treatment and bacteremia

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Y - Public Health agency of Canada Vaccine Preventable Diseases Rubella p91-15, 2007

INFECTION AND IMMUNITY, Jan. 1996, p. 363–365
0019-9567/96/$04.00+0

Vol. 64, No. 1

P55, an Immunogenic but Nonprotective 55-Kilodalton *Borrelia burgdorferi* Protein in Murine Lyme Disease

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Received 31 July 1995/Returned for modification 22 September 1995/Accepted 20 October 1995

Immunization of C3H mice with P55 (previously called S1), a 55-kDa *Borrelia burgdorferi* antigen that is immunogenic after infection, elicited a strong antibody response but did not protect mice against *B. burgdorferi* challenge. Mice immunized with a P55 fusion protein in complete Freund's adjuvant developed anti-P55 antibodies, detectable at a titer of 1:10,000 by immunoblotting. To determine if a protective response had been elicited, P55-vaccinated mice were fed upon by ticks infected with *B. burgdorferi*. The frequency of *B. burgdorferi* infection was similar in P55-immunized and control mice, and spirochetes were not destroyed within ticks that fed on P55-vaccinated mice. P55 is an immunogenic antigen that does not induce a protective response in the vertebrate or invertebrate host.

Lyme disease, caused by the spirochete *Borrelia burgdorferi*, is the most common tick-borne illness in the United States (1). Difficulties in the diagnosis of infection (1), detection of the tick bite (1), and the treatment of antibiotic-resistant cases (20) have stimulated research into the development of an effective vaccine. Several *B. burgdorferi* antigens have been shown to elicit protective responses in animal models. Outer surface protein A (OspA) is the most extensively tested vaccine candidate: active immunization with OspA or the passive transfer of OspA antibodies protects mice from infection with *B. burgdorferi* (4, 6, 18, 21). OspB (7), OspC (14), OspE (11, 13), and OspF (11, 13) also have roles in immunity but are not as effective as OspA (7, 11, 13, 14). OspB- or -C-vaccinated mice and gerbils are partially protected from infection, and the degree of protection may be dependent on the infectious strains of *B. burgdorferi* and the challenge dose of spirochetes (7, 15). Immunization of mice with OspE and -F results in the partial destruction of spirochetes within ticks that engorged on OspE- or OspF-immunized mice but not in absolute protection from infection (13).

Heterogeneity among *B. burgdorferi* antigens may result in vaccine failure. For example, Schaible and colleagues divided *B. burgdorferi* into six groups based on OspA genotypes and demonstrated that passive immunization with sera prepared against one *B. burgdorferi* genotype did not provide cross-protective immunity against others (19). The molecular basis of this lack of cross-protection may be, at least in part, antigenic diversity in the carboxyl terminus of OspA and -B (3, 8, 10, 16, 17), and it can be predicted that similar variability will be reported for OspC, -E, and -F as well. The lack of complete cross-protection suggests that there is a need for the identification of new *B. burgdorferi* antigens that can elicit more broadly protective responses or a protective response that could contribute to a multivalent vaccine.

We have previously cloned a novel *B. burgdorferi* N40 protein, P55 (5), and, as is the case with the antibody response to OspA (the most extensively studied vaccine candidate), antibodies to P55 were present in the sera of some patients with late-stage Lyme disease (17%) (5). Therefore, in this study we investigated the humoral response to P55 in mice infected with *B. burgdorferi* N40 and determined whether active immunization of mice with P55 was protective against *B. burgdorferi* transmitted by *Ixodes dammini* ticks.

Antibodies to OspA appear in the late stage of infection in both mice (2) and humans (9). Therefore, we first determined when C3H mice developed P55 antibodies during the course of infection. We collected sera from three mice each at 7, 14, 21, 28, 90, and 180 days after inoculation with 10^4 *B. burgdorferi* N40 cells subcutaneously, time points representing early- and late-stage infection. For the studies, P55 was expressed as a fusion protein with glutathione transferase (GT), a carrier system that has worked well for OspA, -B, -C, -D, -E, and -F. GT-P55 protein and GT (control) were resolved in 12% sodium dodecyl sulfate-polyacrylamide gels by electrophoresis and transferred to nitrocellulose membranes. Immunoblots were performed by incubating the GT and GT-P55 strips with sera (1:100 dilution) from infected mice. The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Stratagene, La Jolla, Calif.). Immunoglobulin G antibodies to P55 were first detected 90 days after infection (Fig. 1). The band at 80 kDa represented the GT-P55 fusion protein and the band at 60 kDa is a degradation product, consistent with our previous studies with OspB (10). Because antibodies to P55 appeared late during infection, similar to the delayed responses to OspA in mice (2), we then studied P55 as a potential vaccine candidate.

Protection from *B. burgdorferi* infection by immunization of mice with a GT-P55 fusion protein was studied by allowing ticks infected with *B. burgdorferi* to engorge upon P55-vaccinated mice. We chose to study immunity to the tick-mediated transmission because it most closely parallels natural infection. Both ticks infected with *B. burgdorferi* N40 and field-collected ticks from Nantucket were used in the study, thereby representing spirochete populations expressing the homologous P55 protein (N40) and a natural population of spirochetes which are from an area where Lyme disease is highly endemic and which may have variable P55 antigens (5). Groups of female C3H/HeJ mice were immunized subcutaneously with 10 μg of recombinant P55 fusion protein in complete Freund's adjuvant

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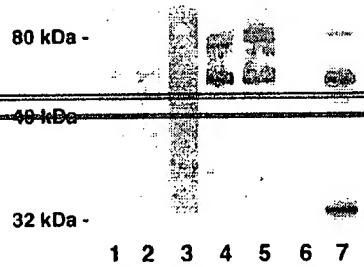

FIG. 1. P55 antibodies in mice infected with *B. burgdorferi*. Purified GT-P55 fusion protein or GT (control) was probed, by immunoblotting, with serum from mice infected with *B. burgdorferi* N40 for various time periods (7 to 180 days). Lanes 1 through 5, GT-P55 fusion protein probed with serum from a mouse infected with *B. burgdorferi* for 7 (lane 1), 14 (lane 2), 21 (lane 3), 90 (lane 4), or 180 (lane 5) days; lane 6, GT probed with serum from a mouse infected for 90 days; lane 7, GT-P55 probed with mouse anti-P55 serum. The band at 80 kDa represents the full-length recombinant GT-P55 fusion protein, and the bands at 60 and 35 kDa are degradation products of the fusion protein.

and given boosters at 14 and 28 days containing the same amount of protein in incomplete Freund's adjuvant (6). Control mice were immunized with 10 μg of recombinant GT in an identical fashion. Fourteen days after the final booster, antibodies to P55 were detectable by immunoblotting at a minimum dilution of 1:10,000. Individual mice were then exposed to five ticks infected with *B. burgdorferi* N40 or five field-collected ticks from Nantucket. This maximized the chances for each mouse to have been bitten by an infected tick. Ticks were allowed to feed to repletion and collected over a water bath as described for examination (13). Mice were sacrificed 14 days after the retrieval of ticks from the water.

B. burgdorferi infection of mice was determined by either culture, amplification of *ospA* by PCR from sera, or the presence of evidence of arthritis and/or carditis. When mice were sacrificed, selected tissues (blood, spleens, bladders, and ear punches) from the animals were collected aseptically, homogenized (spleen), cultured in BSK II media, and examined by dark-field microscopy for spirochetes as previously described (6, 7). *ospA* was directly amplified from sera collected from infected mice according to published protocols (12). DNA extracted from 200 μl of serum was used as the template. Amplified DNA was separated on agarose gels, transferred to nitrocellulose filters, and probed with ^{32}P-labeled *ospA*. Joints and hearts were formalin fixed, paraffin embedded, sectioned, and examined under a microscope for inflammation (6). A mouse was considered infected with *B. burgdorferi* if spirochetes were cultured from tissue, *ospA* was amplified by PCR, and/or disease was evident. Table 1 shows that the rates of *B. burgdorferi* infection were similar in GT- and GT-P55-immunized mice upon which *B. burgdorferi* N40-infected or field-collected ticks engorged, indicating that immunization with P55 was not protective against *B. burgdorferi*. In contrast, a concomitantly performed experiment showed that active immunization with OspA fully protected mice against tick-mediated *B. burgdorferi* infection (data not shown).

We then investigated whether the immune response to P55 could destroy spirochetes within ticks that engorged on GT-P55-immunized mice. For example, it has been shown that immunization with OspE does not elicit protection but is able to induce partial destruction of *B. burgdorferi* within ticks engorged on OspE-vaccinated mice (13). Therefore, spirochetes within the collected ticks were examined as previously described (13). Engorged ticks that fed on GT-P55 or GT immunized mice were collected, kept at room temperature for 10 days, and then dissected. Five microliters of midgut lysates was placed on a glass slide and triplicates of each sample were prepared. The slides were air dried for 1 h and incubated with rabbit anti-*B. burgdorferi* N40 antibodies conjugated with fluorescein isothiocyanate at 37°C for 30 min and washed in H_2O twice. Slides were covered with one drop of mounting medium and examined on a Zeiss Axioskop microscope. Table 1 shows that the numbers of ticks infected with spirochetes were similar in control and P55-vaccinated mice. Moreover, the number of spirochetes in each tick was not significantly decreased in ticks engorged on GT-P55 immunized mice and ranged between 100 and 1,000 *B. burgdorferi* organisms in each specimen sample, as did that within ticks that had engorged on control mice.

Mice infected with *B. burgdorferi* (not hyperimmunized with P55) developed immunoglobulin G antibodies to P55 at 90 days after challenge, indicating that the temporal development of P55 antibodies is similar to the genesis of antibodies to OspA. Moreover, P55 is immunogenic in mice, and active immunization with P55 results in high titers of P55 antibodies (5). However, the immune response to P55 lacks the ability to elicit protection in mice or to destroy spirochetes within engorged ticks. In these studies P55 was expressed as a GT fusion protein, a system that has been effective for the generation of OspA, -B, -E, and -F antibodies, making it unlikely that this specific preparation of P55 resulted in an inability of the mice to make appropriate P55 antibodies. Moreover, Probert and LeFebvre (15) showed that immunization with the recombinant OspD or the recombinant 83-kDa antigen was also not protective. In addition, in contrast to the immune responses elicited by several *B. burgdorferi* outer surface proteins, including OspA, -B, -E, and -F, the immune response to recombinant P55 does not play a role in the destruction of *B. burgdorferi* within ticks or protection of mice against infection. Alternatively, it is possible that the ineffectiveness of P55 as a vaccine reflects idiosyncratic features of the murine model and not an inherent failure of P55 to elicit antibodies that, alone or in combination with complement and/or effector cells, injure or

TABLE 1. Tick-transmitted Lyme borreliosis in mice immunized with GT-P55 or GT (control) proteins

Tick group	Immunogen	No. of mice positive/no. tested			No. of engorged ticks with *B. burgdorferi*/no. tested (%)
		Culture and/or PCR[a]	Disease[b]	Infection[c]	
Infected with *B. burgdorferi* N40	GT-P55	10/17	4/17	10/17	23/40 (57)
	GT	13/17	6/17	13/17	16/47 (34)
Field collected (Nantucket)	GT-P55	4/10	2/10	4/10	1/8 (13)
	GT	3/10	2/10	4/10	2/17 (12)

[a] Positive by culture (blood, bladder, spleen, and skin) and/or PCR (blood).
[b] Arthritis and/or carditis.
[c] Positive by culture and/or PCR, positive for disease, or both.

kill the spirochete. It can be predicted that even though many antigens, such as P55, will be cloned and found to be immunogenic and recognized by murine and patient sera, the majority are not going to elicit protective immunity in murine model.

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VACCINES

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29 New Technologies for Making Vaccines

The development of new techniques in molecular genetics has expanded the number of approaches that can be used for making vaccines. In some cases, established vaccines can be improved or their supply increased. In other cases, new vaccines can be developed that have not been feasible through the application of old technologies. In this regard, it is worth considering first the broad categories into which vaccines can be divided. "Live" vaccines are defined by the ability of the vaccine strain, i.e., of the virus, to replicate within the human host. Conversely, "killed" vaccines ("non-live" may be more accurate, even though most scientists use the term "killed") are unable to replicate or infect the host. Table 29–1 summarizes the salient features of these two categories of vaccines.

Live Vaccines

Live vaccines are attenuated with respect to their ability to cause disease, meaning that they are less likely to cause clinical illness than the natural disease-causing agent. By virtue of their ability to undergo limited replication in the host, such vaccines, typically viruses, often induce cell-mediated (T cell) immunity in addition to antibody-mediated (B cell) immunity. As a result of such a broad spectrum of immunity as well as re-exposures to the virus which silently boost immunity, protection following a single inoculation with a live attenuated vaccine often lasts a lifetime. However, the ability of the live vaccine to replicate can be detrimental; being genetically plastic, a replicating virus can revert to a more pathogenic form and cause adverse reactions in a vaccinee or a contact of a vaccinee. Sufficient data must be obtained in animal studies as well as in clinical studies to rule out the possibility of reversion.

A number of strategies have been employed for developing live viral vaccines that are attenuated, as summarized in Table 29–2.

Several of these approaches were possible before the development of modern techniques in recombinant DNA (rDNA) technology which enable the manipulation of viruses on the molecular level. These classic approaches, which utilize routine techniques in cell culture, include attenuation in cell culture, selection for temperature-sensitive or cold-adapted viruses, isolation of closely related viruses from other species and selection for reassorted viruses from the progeny of an infection by two parental viruses. (These strategies are discussed in greater detail elsewhere with respect to particular vaccines.)

The ability to alter directly the structure of viruses on the molecular level is enabling scientists to design attenuated vaccines rather than forcing them to rely upon phenotypic selection and upon chance to provide the only mechanisms for viral change. Through techniques of viral genetics and DNA sequence analysis, it is possible to identify those regions in the viral genome where alteration can contribute to the attenuation of viral pathogenicity. This rDNA technol-

Table 29–1. General Characteristic of Vaccines

"Live" Vaccines
Attenuated with respect to pathogenicity
Cell-mediated immunity in addition to humoral immunity
Longer-lasting protection
Tendency to reactogenicity
Ability to revert
"Killed" Vaccines
Nonreplicating
Noninfectious
Lower reactogenicity
Need for boosters
High purity

Table 29–2. Strategies for the Development of Attenuated Live Viral Vaccines

"Classic" Approaches
Modified by passage in cell culture
Variant viruses from other species
Temperature-selected mutants
Reassorted genomes
"Molecular" Approaches
DNA modification mutants
Recombinant viruses

ogy allows such regions to be altered or deleted and introduced into the genome of a wild-type virus, thus leading to the production of an attenuated virus. This approach is presented in the schematic in Figure 29–1.

The salient feature of this approach is the deliberate construction of an attenuated virus that is unlikely to revert to a more pathogenic form. This construction is made possible by deleting a portion of a key region of the genome in such a way that reversion is ruled out. This approach first was applied successfully by Kit and coworkers to the attenuation of pseudorabies virus, thus leading to the creation of a safer vaccine for the prevention of a severe disease in pigs.[1] This is the first genetically altered live vaccine that was licensed for use in any species. A related approach is being taken for poliovirus and is applicable to other vaccines for humans.[2]

A second approach is the genetic alteration of a live virus to function as a vector, *i.e.*, carrier, for other genes. This approach enables the recombinant virus to function as a vaccine for two or more infectious agents in a single inoculation. This technology first was applied to vaccinia virus.[3, 4] Prior to this application, wild-type vaccinia virus had been used for the worldwide eradication of smallpox and is the prime example of a variant virus from another species used as a vaccine for humans. A region of the genome of vaccinia virus was identified as nonessential for viral replication by the general approach outlined in Figure 29–1. Within a plasmid containing this nonessential region, a gene encoding a surface protein of another pathogen was inserted (Fig. 29–2). This recombinant plasmid was introduced together with wild-type virus into cells in culture, resulting in the creation of a recombinant virus that carries the foreign gene.

For insertion into a virus vector, a gene is selected that encodes an immunogen, usually a surface protein, of a virus or a microbial parasite. In order for this strategy to be effective, the presentation of this immunogen during the course of viral replication should result in a protective immune response directed to the antigen and, therefore, the pathogen. Recombinant vaccinia viruses have been derived that express immunogens for hepatitis B virus, herpes simplex virus, influenza virus, rabies virus, Epstein-Barr virus and respiratory syncytial virus. Some of these recombinant viruses have shown promise in animal studies. A similar approach has been taken with respect to the genetic engineering of two human herpesviruses as viral vectors, herpes simplex virus[5] and varicella-zoster virus.[6]

Table 29–3 outlines several points that are important to the safety and efficacy of such live recombinant vaccines. A nonessential (i.e., not required for viral replication) region for the insertion of a foreign gene often can be used that will result in the attenuation of viral pathogenicity.[7] Multiple foreign genes can be inserted into a single viral genome, resulting in an immune response against multiple pathogens.[8] The level of expression of the foreign protein should be high enough to elicit effective immunity. The parental (vector) virus should be tested extensively; its use as a vaccine should be free of side effects. In that regard, the use of the smallpox vaccine strain of vaccinia virus has raised concern with respect to the neurological and dermatological sequelae observed in small numbers of vac-

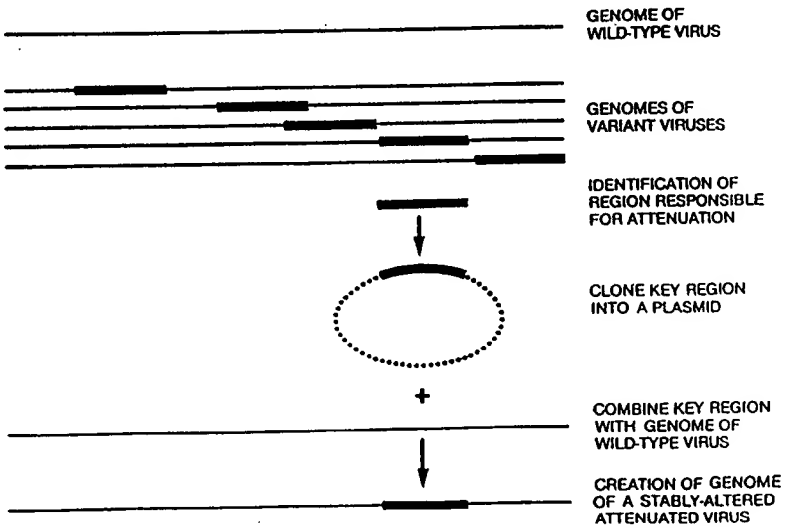

Figure 29–1. Attenuation of viruses using modern techniques in molecular biology.

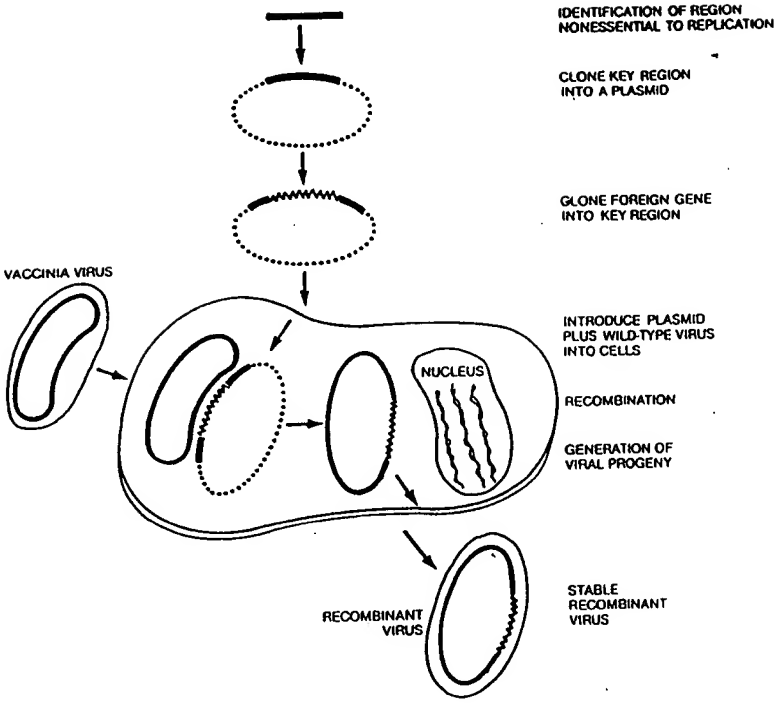

Figure 29–2. Creation of recombinant vaccinia viruses carrying genes that encode immunogens of other pathogens.

cinees. The host range or tissue tropism of the recombinant virus should not be altered significantly compared with that of the vector virus. The effects of viral infection upon the replication and structure of host cells should be studied closely. Since vaccinia virus encodes a protein with significant homology to transforming growth factor-α and to epidermal growth factor (EGF) and since the virus infects cells through the EGF receptor,[9] which is itself highly homologous to the *erb*-B oncogene, there is concern that infection with vaccinia virus may be mitogenic (stimulates growth or division of infected cells). Finally, while some recombinant vaccinia viruses have shown promise in preclinical testing in models of efficacy in animals, only clinical trials and testing of protective efficacy in humans, still awaited, will permit a complete assessment of the utility of such vaccines.

Table 29–3. Considerations in the Safety and Efficacy of Recombinant Live Vaccines

Safety
Extensive testing of parental virus
Stable attenuation of parental virus
Insertion point for the foreign gene
Host range of the recombinant virus
Biology of the cellular receptor for the virus
Efficacy
Multiple foreign genes in a single virus vaccine
Level of expression of foreign protein
Clinical testing

Killed Vaccines

In contrast to live vaccines, killed vaccines do not replicate in the host. Consequently, killed vaccines are often less efficient in the induction of cell-mediated immunity. In order to achieve complete and long-term protection, booster inoculations are required. Furthermore, the greater antigenic mass required for a killed vaccine to be effective, when compared with the antigenic mass for a live vaccine, raises issues of purity. Since they do not replicate, killed vaccines cannot revert to cause clinical disease. Several strategies have been used to develop killed vaccines, as summarized in Table 29–4.

The classic approaches, which employ techniques of biochemical purification and biophysical inactivation, include physical inactivation of whole viruses or bacteria, utilization of inactivated toxoids from bacteria, purification of mon-

Table 29–4. Strategies for the Development of Killed Vaccines

"Classic" Approaches
Killed whole pathogens
Toxoids from pathogens
Purified surface components
Conjugated surface components
"Molecular" Approaches
Recombinant-derived proteins
Synthetic peptides
Anti-idiotypic antibodies

omeric or aggregated surface components of viruses or bacteria and conjugation of surface components of bacteria to other molecules. (These strategies are discussed in greater detail elsewhere.)

The techniques of rDNA have revolutionized biomedical research. They make it possible to identify the gene encoding any protein of interest and to insert that gene into a host cell in such a way that the cell can produce large amounts of the particular protein (Fig. 29–3).

This technology is directly applicable to the development of vaccines. The key to the problem is the identification of that protein component of a virus or microbial pathogen that itself can elicit the production of protective antibodies, such antibodies having the capacity to neutralize infectivity and thus protect the host against attack by the pathogen. The protein then defines biochemical tools for research (e.g., antibodies and amino acid sequences), which are useful for the identification and cloning of the gene encoding that protein. Ultimately, the gene is placed into a host cell in a configuration that will result in synthesis by the host cell of large amounts of the particular immunogenic protein.

The initial application of rDNA technology to the development of vaccines for humans was for the vaccine to prevent infection by hepatitis B virus (HBV). A safe and effective vaccine, consisting of particles of the surface antigen of HBV (HBsAg) has been prepared from human plasma. In order to expand the available supply of vaccine, scientists turned to rDNA-technology for vaccine production. The process was initiated by the identification of the gene encoding HBsAg and the insertion of that gene into various host cells. Recombinant yeast synthesize large amounts of particles of HBsAg that are morphologically (Fig. 29–4) and immunologically highly similar to the plasma-derived HBsAg.[10]

Recently, the yeast-derived HB vaccine produced by Merck, Sharp & Dohme became the first rDNA-derived vaccine of any type for humans ever to be licensed anywhere in the world. This prototype vaccine offers hope for the development of a new generation of vaccines, including ones for diseases such as malaria[11, 12] and leprosy[13] for which vaccines cannot be made using classic technologies. The development of recombinant vaccines ultimately may be facilitated by the application of new techniques for the enhancement of the immunogenicity of isolated proteins; one such technique is hydrophobic aggregation.[14] However, because of the biology of the disease or the nature of the immune response induced by the vaccine, it is important to realize that recombinant vaccines do not always provide the solution to the problem of prevention of an infectious disease.

There are a large number of host cells that can be utilized for the production of rDNA-derived proteins. The most common host cells have been bacteria (*Escherichia coli*), yeast (*Saccharomyces cerevisiae*) and mammalian cells (Chinese hamster ovary, monkey kidney). Recently, scientists

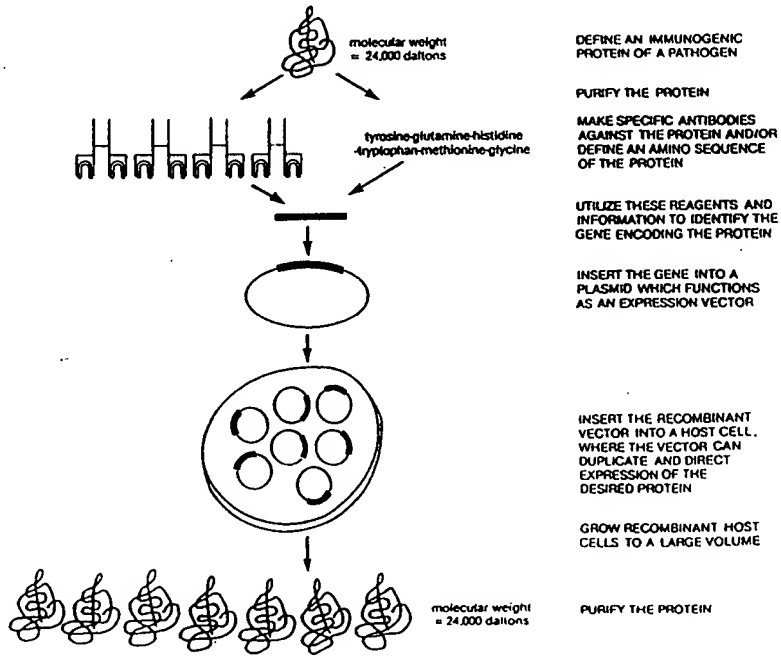

Figure 29–3. The use of recombinant DNA (rDNA) technology to express large amounts of a desired protein.

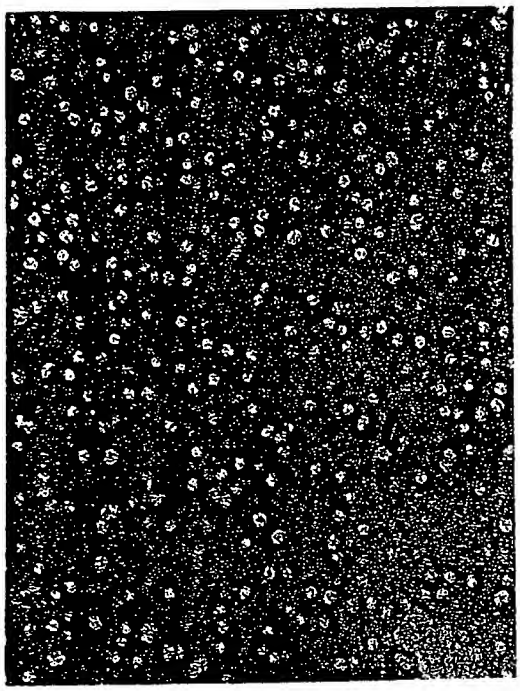

Figure 29–4. Electron micrograph of particles of HBsAg produced by recombinant yeast (165,000X). (Courtesy of B. Wolanski, Merck Sharp & Dohme Research Laboratories.)

have diversified to the use of other bacterial (*Bacillus subtilis*), fungal (*Aspergillus nidulans*) and higher eukaryotic (insert) cells. All these systems can be judged by a wide range of criteria relating to desirable traits of either the protein product or the host cell as well as to safety considerations (Table 29–5).

The most commonly employed expression systems can be evaluated relative to one another

Table 29–5. Expression Systems for rDNA-derived Proteins

Desirable Traits of the Product
High yields (commercial)
Stability of yield with scale-up of cells
Inducible expression
Secretion
Post-translational modifications (consistent with immunogenicity)
Glycosylation
Phosphorylation
Amidation
Carboxylation
Hydroxylation
Proteolytic processing
Desirable Traits of the Host Cells
Ease of scale-up
Consistency of performance
Lack of oncogenic elements
Rapid division
Safety Concerns
Heterologous protein contaminants
Biology of cell substrate
Residual DNA (oncogenesis)

*Scheme to take cultures from bench to large-scale fermentation or purification.

with respect to each of these criteria (Table 29–6). These criteria fall into three groups, which roughly discriminate between the microbial (yeast and bacteria) and mammalian expression systems as follows:

1. The microbial systems are more productive and consistent in overall performance than the mammalian ones.
2. Mammalian cells provide for post-translational modifications that often resemble more closely those in the viral agent than those provided by microbial cells.
3. With few exceptions, serially propagated

Table 29–6. Comparison of Commonly Used Expression Systems for rDNA

	E. coli (Bacteria)	*S. cerevisiae* (Yeast)	Chinese Hamster Ovary (Mammalian Cells)
Yield of product	+ + +	+ + +	+
Ease of scale-up	+ + +	+ + +	+
Stability of yield with scale-up	+ + +	+ + +	+
Inducible expression	+ + +	+ + +	+
Consistency of performance	+ + +	+ + +	+
Secretion	+	+ +	+ + +
Glycosylation	–	+ +	+ + +
Proteolytic processing	–	+ +	+ + +
Other modifications	–	+ +	+ + +
Biology of cell substrate	+ +	+ + +	+
Heterologous protein contaminants	+ +	+ +	+
Residual DNA	+ + +	+ + +	+

+ + + = most acceptable
+ + = acceptable
+ = least acceptable
– = absent

mammalian cells, unlike microbial cells, are considered "transformed," meaning that they are more susceptible to oncogenicity in experimental animals.

Furthermore, for expression of rDNA, mammalian cells often utilize genetic elements derived from oncogenic or latent viruses, while microbial cells do not utilize such elements. These perceived safety concerns must be addressed regarding the use of mammalian cells as an expression system for recombinant vaccines.

These relative evaluations represent generalizations from a large number of studies in the different systems and should be considered whenever an expression system is utilized. Nevertheless, each attempt at expression must be evaluated individually, and there are probably as many exceptions as there are rules in the "expression game"!

The use of synthetic peptides as vaccines involves the use of short segments of a protein molecule, rather than the entire molecule, as the immunogen. Some peptides are able to induce antibodies that can react with the whole protein as well as with the peptide per se.[15] The discovery process for formulating synthetic peptide antigens begins by defining the gene encoding the immunogenic protein (see Fig. 29–3), then branches off by exploiting the DNA sequence of the gene to define the amino acid sequence of the protein and to predict which regions of the protein might be immunogenic (Fig. 29–5). Once defined, peptides can be synthesized chemically[16] and formulated into synthetic vaccines.

This approach first was applied to the development of vaccines for humans by synthesizing portions of the HBsAg polypeptide.[17] In theory, the approach is technically versatile and lends itself to the production of well-defined vaccines. However, in practice, the approach has several shortcomings relative to the use of whole proteins. In general, the antibodies elicited by an intact protein crossreact more effectively with both the protein and the pathogen on which it resides than do antibodies elicited by a synthetic peptide. Furthermore, such antibodies bind with higher affinity and are present at a higher titer than are those elicited by the peptide. Thus, the duration of the immune response stimulated by a synthetic peptide is inferior to that stimulated by a whole protein. At minimum, a complete cocktail of synthetic peptides may be required as well as an improvement in methods for the enhancement of immunogenicity by covalent conjugation onto carrier proteins. It may be that synthetic peptides, however tailored, cannot mimic all the conformations assumed by the

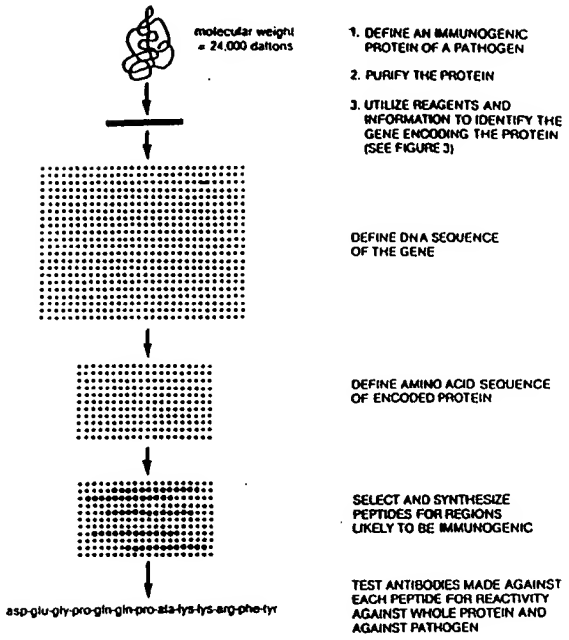

Figure 29–5. Defining immunogenic peptides from immunogenic proteins.

intact protein that are critical for immunogenicity. Furthermore, immunogens often have complex chemical structures, e.g., sugars and lipids, which cannot be specifically applied to a synthetic peptide. However, synthetic peptides may be useful for the priming of an immune response, as first demonstrated for poliovirus.[18]

A third novel strategy for the formulation of killed vaccines is the use of anti-idiotypic antibodies (anti-antibodies), whose existence and function in the regulation of the immune response first were articulated by Jerne.[19] Since antibodies bear a structural image of the primary antigen at the antigen-combining site (idiotype), antibodies to antibodies (anti-idiotype) have an antigen-combining site that is structurally similar to the antigen (Fig. 29–6). Thus, inoculation of the anti-idiotypic antibody functions as a vaccine by inducing an anti-anti-idiotypic antibody which in principle should be identical to the first antibody.[20]

This approach has been applied to formulating a vaccine for hepatitis B.[21] While this strategy clearly warrants further study, it suffers from two potential drawbacks. Since the immunogen is an antibody, which is structurally related to naturally occurring human antibodies, problems related to antigenic sensitization must be addressed. In addition, the images borne by anti-idiotypic antibodies are structurally analogous to peptide domains on the surface of the pathogen rather than to whole proteins. Therefore, such

phisticated analytical tools of molecular biology and immunology, vaccines derived from the newer technologies are receiving closer scrutiny at the regulatory and clinical levels than have vaccines derived from more classic strategies. This trend is expected to continue and represents a formidable barrier for manufacturers to hurdle with respect to the licensing of safe and effective vaccines. As with any technology, there is a learning curve for both manufacturers and regulatory agencies.

A major challenge facing manufacturers and society in the United States is the profound increase in litigation over adverse experiences related to vaccines. The most dramatic manifestation of this litigation is the increased expense and intermittent unavailability of product liability insurance to the three United States–based manufacturers of the vaccine for pertussis, thus resulting in the temporary withdrawal of products of two of these firms from the market and the tripling in the price of the vaccine. Medically, one may find a situation in which the general welfare of the pediatric population may be at significant risk to whooping cough. This problem could have tragic consequences for society and become a severe disincentive for the development of vaccines by means of new technologies. It is hoped that, while legislative remedies to this severe problem are being addressed, research scientists and medical researchers will continue to receive as much support as possible in the pursuit of new technologies, since the vaccines that result represent the most cost-effective products for the eradication of infectious diseases.

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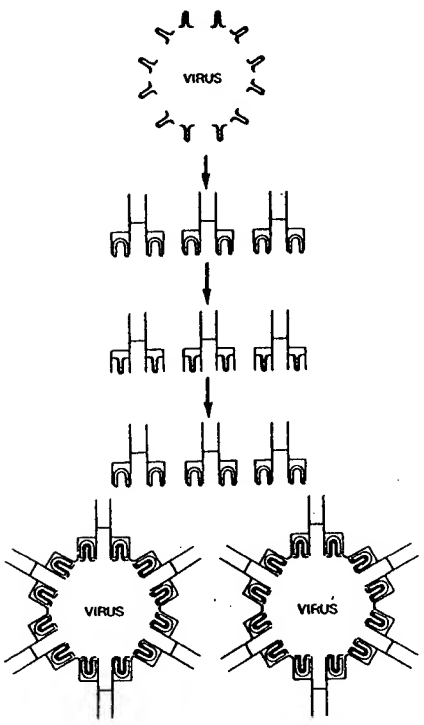

Figure 29–6. Strategy for the use of anti-idiotype antibodies as yaccines.

vaccines might elicit an immune response which is more antipeptide-like in nature rather than antiprotein-like, as discussed previously.

The progression of a vaccine candidate from the laboratory to the marketplace is long and arduous, often taking 10 years from the time of its initial discovery and characterization. Vaccines made by means of new molecular technologies are being developed rapidly. Of these, only rDNA-derived proteins have gone as far as human clinical trials, much less having become a licensed product as in the case of yeast-derived HBsAg. Representatives of such vaccines are listed in Table 29–7, along with others derived by older types of technologies.

With the development of the increasingly so-

Table 29–7. The Progression of Human Vaccines Made by Different Technologies Toward Becoming Licensed Products

	Preclinical Testing	Clinical Testing	Licensed Product	Examples
Live Vaccines				
Classic Strategies				
Modification in cell culture	X	X	X	Measles, mumps, rubella
Variants from other species	X	X	X	Smallpox (vaccinia), rotavirus
Temperature-selected mutants	X	X		Influenza
Reassorted genomes	X	X		Rotavirus
Molecular Strategies				
DNA modification mutants	X			Poliovirus, *Salmonella, Shigella*
Recombinant viruses	X			Vaccinia, herpes simplex, varicella-zoster
Killed Vaccines				
Classic Strategies				
Killed whole pathogens	X	X	X	Pertussis
Toxoids from pathogens	X	X	X	Diphtheria, tetanus, cholera
Purified surface components	X	X	X	Hepatitis B
Conjugated surface components	X	X		Meningitis (*Hemophilus influenzae* b)
Molecular Strategies				
Recombinant-derived proteins	X	X	X	Hepatitis B
Synthetic peptides	X			Hepatitis B
Anti-idiotypic antibodies	X			Hepatitis B, rabies

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Status of immune-based therapies in HIV infection and AIDS

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SUMMARY

As our understanding of HIV disease pathogenesis progresses, approaches to immune-based therapy are evolving. Initial therapies aiming to alter immune function in patients with HIV infection have had mixed results. Clinical benefit in the trials so far has not been dramatic, although the studies are still at an early stage, and the correct protocols for the various agents or combinations of agents have yet to be established. As might be expected, where apparent benefit has occurred, it has been more obvious in those patients whose immune system was still intact.

Keywords cytokines growth factors immune modulators vaccines antibodies HIV

INTRODUCTION

Immune-based therapies are of great interest in AIDS, because specific immunity is the normal means of containment and eradication of infectious agents, and because HIV infection is characterized by immune deficiency. Many approaches to preserving and restoring immune competence have been explored and those reviewed here are grouped as cytokines and growth factors, immune modulators, receptor-directed therapy and specific immune interventions (antibodies and vaccines). Many features of the reports are summarized in Table 1.

CYTOKINES AND GROWTH FACTORS

IL-2

Administered to patients with AIDS or ARC-induced transient or no detectable immunological improvement; administration of IL-2 in conjunction with zidovudine (ZDV) to asymptomatic patients induced early (transient) and, in some, prolonged increases in CD4 T cells without an increase in p24 antigenaemia [1]. Patients with minimal disease responded well to IL-2 with increased MHC-restricted cytotoxicity to HIV-bearing cells [2]. There was also an expansion of natural killer (NK) and lymphokine-activated killer (LAK) cytotoxicity and non-MHC-restricted, cytolytic activity which reflects antibody-dependent cellular cytotoxicity (ADCC) [3]. Delayed-type skin hypersensitivity responses were decreased at higher doses of IL-2 [1]. Overall, those with advanced disease showed much less immune augmentation in response to IL-2 administration.

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Interferon-alpha (IFN-α)

IFN-α has no effect as an anti-HIV agent unless there is residual immune competence in the treated patient. About 40% of patients with Kaposi's sarcoma (KS) respond to IFN-α with regression of KS lesions [4–7]. Those with CD4 T cell levels below 150/mm^3, however, have a very poor likelihood of responding. IFN-α was given at the maximum tolerated dose, which caused moderate symptoms in almost all recipients, including leukopenia and lymphopenia. Zidovudine (ZDV) has a pre-transcription effect and IFN-α acts post-transcription on HIV mRNA (*in vitro* studies). A combination of AZT and interferon therapy for a minimum of 12 weeks in an initial trial showed only a slight decline in CD4 cells and a decrease in serum p24 antigen.

Interferon-beta (IFN-β)

IFN-β given intravenously has a similar effect and uses the same receptor as IFN-α, but may have less haematopoietic suppressive action [8]. Subcutaneous administration of IFN-β in similar doses has less anti-tumour and less anti-KS effects. An anti-viral effect (reduction in serum p24 antigen) was seen only in those with normal β2-microglobulin levels prior to therapy or who responded with an elevation of β2-microglobulin during IFN-β administration. A response was evident only in those with relatively competent immune systems.

Interferon-gamma (IFN-γ)

Administration of IFN-γ in patients with AIDS showed no evident clinical benefit, although there was some increase in monocyte activation [9]. IFN-γ, however, may be effective in inhibiting metabolic pathways of intracellular organisms such as *Toxoplasma gondii* and is beginning to be tried in combina-

Table 1. Summary of results of immune-based therapies

Cytokines/lymphokines	Clinical	Immunological changes	Other
IL-2	Improvement in patients with CD4 >400/mm^3; high dose required	Increased in CD4 with ZDV Increased NK & T cytotoxicity	
Interferon-alpha	More and KS effect with high dose, flu-like symptoms very common (40–60%); better response with CD4 >150/mm^3	CD4 percentage increase in some	
Interferon-beta	Similar to IFN-α but less anti-KS effect	Increase in serum β2-microglobulin	
Interferon-gamma	Possible effect to limit opportunistic infection (OI)	Increased macrophage-peroxidase and HLA-DR	
GM-CSF, G-CSF	Lowest effective dose not established. Reduction in infections uncertain	Pokeweed mitogen *in vitro* responses increased	
Immune modulators: generally trials during ARC or AIDS			
Diethyldithiocarbamate	Reduced progression and or frequency of OI reported	None documented for lymphocytes. Free radical scavenger probably effects monocytes	Effects in minimal or early disease not known
Isoprinosine	Many negative studies. One good study positive for reduced occurrence of OI	No changes confirmed	
Receptor-directed therapy			
Soluble CD4	35 min half-life. No certain clinical benefits	No changes documented	Strains of HIV-1 freshly isolated from patients are 100–1000-fold less sensitive to rsCD4 than one laboratory strain.
CD4 adhesins (IgG derived)	1 day half-life. No certain clinical benefit	No changes documented	Adhesins reduce monocyte infection *in vitro*
Antibody therapy			
Immune serum gamma globulin	No certain clinical change	Increased in vitro/proliferative responses in some patients	
Murine monoclonal (anti-gp160)	Pilot study; no clinical benefits	No change in CD4. Improved *in vitro* proliferative response	Improved B cell functions
Vaccine in HIV-infected persons			
Recombinant gp160 with Freund's incomplete adjuvant	Specific immune responses when CD4 T cells over 600/mm^3. Soreness at site of infection	Specific antibodies and cell mediated immunity to vaccine/ in most; stabilization of CD4 levels in some	Six doses more effective than three. Only gp160 antigen specificities
Killed HIV vaccine (gp160 antigen depleted)	Immune response better with higher CD4 levels Soreness at site of infection	Generally similar, except for specific immunity for different antigens.	Most HIV antigens except gp160.

KS, Kaposi's Sarcoma; ZDV zidovudine.

tion with antibiotics to enhance host defence against opportunistic infections.

Granulocyte (G-CSF) and granulocyte–monocyte (GM-CSF) colony-stimulating factors

These substantially increased circulating neutrophils [9]. Reduced doses still had the desired effect and the lower limit of effective dose has not yet been established. These studies indicated a reduction in the occurrence of infection. One of the complications from GM-CSF administration is dermatitis, sometimes of the eosinophilic type, that requires cessation of administration. The mechanism of this complication has not been defined.

IMMUNE MODULATORS

Diethyldithiocarbamate (DTC)

Extensive work in a retrovirus-induced murine immune deficiency disease indicated that DTC prevented disease progression [10]. DTC acts as a free radical scavenger and may modify some macrophage functions advantageously. Three studies of DTC were done in HIV infection in France, Germany and the United States. The latter was a randomized, placebo-controlled trial of DTC [11]. A lack of clinical progression (e.g. reduced occurrence of opportunistic infection) was reported in all three trials. More recent studies, however, failed to substantiate these findings and the Institute Merieux has withdrawn DTC from further trials.

Isoprinosine

Isoprinosine is metabolized *in vivo* to several compounds with uncertain biological effects. Data in a large, well-controlled, cooperative clinical trial in Scandinavia reported benefit which occurred in those with more advanced disease, e.g. those with advanced ARC or AIDS [12]. The main effect seemed to be on the occurrence of *Pneumocystis Carinii* pneumonia (PCP). However, this work has been subject to some criticism; there was some difference in the incidence of candida infection between the two groups before treatment, e.g. a higher incidence in the control group and the serum p24 antigen level did not change. A follow-up study is indicated with a design including ZDV with and without isoprinosine, which would include immunological and virological evaluation, as well as clinical endpoints. The Scandinavian study, however, does not agree with negative findings in several other carefully done clinical trials of isoprinosine. Extensive immunologic evaluations were carried out during isoprinosine administration in a study in the U.S. and Britain (M. Loveless, personal communication) but treated subjects were not different from controls in CD4 numbers, in NK activity, proliferative responses, IL-2 production or in clinical response. In a 6-month study at Northwestern University there was no clinical or immune change [13].

RECEPTOR-DIRECTED TREATMENT

Soluble CD4

The original rationale was to block viral infection by binding the virus to soluble CD4 molecules. Also, blocking the access of gp120 to cell surface CD4 could protect against immune-mediated cytolysis by ADCC or CTL cytotoxic mechanisms, and against syncitium formation between uninfected $CD4^+$ cells and those expressing surface HIV gp120. Several trials were conducted with escalating doses of soluble CD4 [14]. Repeated intramuscular injection of 1, 3 and 10 mg produced levels in serum in the range of those which inhibited viral replication *in vitro*. There was, however, little evidence of inhibition of viral replication. The *in vitro–in vivo* discrepancy may be explained in part by the observation that viruses isolated from patients receiving soluble CD4 were 2 logs less sensitive to soluble CD4 than are laboratory strains of HIV [15]. Recent studies indicate that the differences between laboratory and clinical strains of HIV to soluble CD4 may relate to stripping of gp120 from clinical versus laboratory strains of the virus [16].

*Immunoadhesins **(CD4–IgG)*

By combining CD4 molecules with constant domains of IgG molecules [17], it has been possible to increase the half-life substantially (from 35 min for soluble CD4 to about 1 day). CD4-immunoglobulin complex was given intravenously and intramuscularly and various schedules explored from daily to twice a week with 1 mg/kg per day as the peak dose so far.

There was little transfer into the CSF. CD4 levels did not change and clinical improvement was not noted. There was a tendency to reduced p24 antigen levels and reduced viral load without statistical significance. Higher doses may eventually get over a threshold, allowing this agent to become effective against, at least, some viruses. However, it should be noted that the same differences in susceptibility between laboratory propagated and clinical isolates of HIV to soluble CD4 preparations are exhibited by the CD4/IgG immunoadhesin [15].

SPECIFIC IMMUNE INTERVENTIONS: ANTIBODY STUDIES

Immune serum and gammaglobulin

IgG preparations from pooled sera allow the administration of larger amounts and with better standardization than does immune sera from single individuals. IgG prepared at Abbott Laboratories from HIV^+ donors was shown to be free of hepatitis virus and live HIV. High titres of antibodies to HIV p24, gp41 and gp120 were present. Neutralization of HIV IIIB and MN strains was documented. A phase 1 trial was done with 12 AIDS patients taking ZDV (at least 300 mg/day for at least 90 days) who appeared to be clinically stable. The individuals who received two 50 mg HIVIG/kg doses followed by two 200 mg/kg doses given at 1-month intervals, i.e. for a total of 4 doses, had no severe side effects from the HIVIG. CD4 T cells and serum β2-microglobulin levels did not change and clinical change was not noted in these first patients with advanced disease.

Murine MoAbs to gp120 core loop

These MoAbs have been evaluated in Sweden (Eric Sandström, personal communication). One MoAb that neutralized almost all available HIV strains was purified by affinity chromatography and 125 mg were given every 2 weeks for 3 months with an *in vivo* titre of about 1 : 50 000 in the recipients. The infusions induced fever and chills which were generally well tolerated. CD4 T cell and serum p24 antigen levels varied in the recipients without a certain trend. Viral isolations remained positive.

Murine anti-gp160 administration was associated with an increase in proliferative response to mitogen. Since gp160 has been shown to be immunosuppressive, it is possible that this antibody cleared circulating gp160 and reduced the immune depressive action and allowed the lymphocytes to function better. Also, the improved B cell effect could be from removal or reduction of immmunosuppressive factors. However, 80% or more of HIV^+ individuals have antibodies capable of blocking gp160 binding to CD4 T cells *in vitro*. Thus, it is not clear how adding these additional antibodies would make a difference.

VACCINE ADMINISTRATION TO HIV-INFECTED INDIVIDUALS

Vaccine administration is based in part on the premise that the prolonged course of HIV infection is attributable to specific immune responses that develops to the virus shortly after exposure and that these responses are temporarily effective but eventually fail. An additional premise is that the vaccine can be used to induce protective immunity to augment natural host immunity. Vaccination treatment of herpes simplex virus (HSV) infection has been reported in the pig by using IL-2 incorporated into liposomes along with recombinant HSV [18]. Immunization given post-infection induced both qualitatively and quantitatively different immune responses from those induced by natural infection. Furthermore, the course of the herpes simplex genitalis infection was modified [18].

HIV gp160 vaccine

This vaccine was administered by Robert Redfield and colleagues using recombinant gp160 (MicroGeneSys) in 30 patients with Walter Reed stages 1 and 2 HIV infection (e.g. relatively early in the disease course) [19]. A responder was defined as one

who had repeatedly demonstrated antibody against gp160-specific antigens and a demonstrable proliferative response against gp160 that was associated temporally with immunization.

Increased antibody to HIV was demonstrated following immunization. There was an increase in already existing antibodies as well as appearance of antibody specificities not detected during natural infection. Even though the antibody induced by gp160 was derived from HIV strain IIIb, there was an increase in neutralizing activity against the MN and RF strains.

Increased cellular immunity was found in many individuals after vaccination. There were different patterns of response in terms of the strength of response to different gp160 epitopes. These differences in response and potential relationship to clinical course provide special opportunities to relate HIV-specific immune responses to protection in existing infection. The subjects receiving gp160 vaccine did not show excessive reductions in CD4 cells or of delayed-type hypersensitivity reaction or increased viral production.

The group who had the more vigorous specific immune response to the vaccine had little or no decline in the CD4 T cell levels. In contrast, those who responded poorly to immunization did show the expected fall in CD4 T cell levels. The response rate was substantially higher in the group that received 6 doses of vaccine (about 65%) in contrast to those that received three doses of vaccine (about 40%).

HIV (gp120 depleted) vaccine

This vaccine was prepared by Jonas Salk and colleagues [20] has been evaluated by Alexandra Levine and associates at the University of Southern California [21]. HIV from a cultured supernatant is inactivated by propiolactone and ^{60}Cobalt irradiation. The gp120 envelope protein is largely lost but the remaining structural proteins are retained. Thus, this vaccine is in many ways the converse to the gp160 vaccine reported by Redfield *et al.* [20]. The HIV preparation is dissolved in saline and emulsified in Freund's incomplete adjuvant and given by intramuscular injection at 100 mg/dose [21]. After chimpanzee studies showed that the material was not infectious in tissue culture but immunogenic for HIV antigens (except gp160) in animal studies, clinical trials were instituted in HIV$^+$ patients with lymphadenopathy. Initial studies revealed no toxicity. Median follow-up time was 2·3 years. The vaccine was given every 3 months for the first year and then every 6 months. Transient local effects were seen in a third of the patients with soreness at the site of infection. A total of 86 have received this vaccine. Sixty per cent of ARC patients became HIV skin-test positive and all were negative at baseline. Most of the individuals had extensive follow up before treatment and more gained than lost weight when treated. CD4 T cell levels remained stable over time in one group and in another lost a mean 100/mm^3 over a 2-year period. Those who developed skin test positivity had a higher CD4 T cell level at entry and maintained the level. This dependence on immune competence is similar to the findings reported above by Redfield *et al.* [20] with a very different vaccine. Serum β2-microglobulin did not change during the course of the study in asymptomatic or ARC patients. Measurements of p24 antigen and quantitative polymerase chain reaction (PCR) tests were inconclusive.

DISCUSSION

The need for better anti-retroviral and immune reconstitution therapies in HIV infection is well recognized. Since HIV infection results in immune activation, there is a need to distinguish between beneficial and potentially harmful immune activation. Activation causing viral replication, increased viral load and disease progression contrasts with potentially beneficial immune activation, such as that promoting specific humoral and cellular immunity which provide protection against HIV. The question of whether administration of cytokines such as IFN-α to individuals who are already making substantial amounts and have elevated blood levels has been debated. In the case of IFN-α there is evidence [5, 6] that KS may respond to the higher levels, especially if the immune system is still functioning fairly well [7]. There may be pharmacological as well as physiological aspects to the administration of some compounds. With several cytokines, the effects depend on the stage of the illness, e.g. on residual immune competence as noted for IL-2 and IFN-α. A difference between *in vivo* and *in vitro* effectiveness has been emphasized since many compounds have a short activity *in vivo*. Circulation through the liver and the kidney *in vivo* can reduce the persistence of therapeutic agents markedly.

Do specific therapies such as antibody administration cause adverse change in HIV viral characteristics? Data from the use of antibody treatment has not been reported. Hyper-immunization with gp120 did not induce viral changes. A second question relates to the point that antibodies are being tried so far only in patients who have quite advanced disease. Preliminary data with antibodies in paediatric studies, as well as the cytokine and vaccine studies noted above, indicate that many treatments might be more effective with less advance disease or less aggressive disease and with more intact immune systems.

Progressively lower doses of lymphokines, cytokines and related factors have been suggested for future trials. Observations with G-CSF indicated that initial doses could be reduced substantially without loss of effectiveness. This is an important shift in therapeutic concept away from that generally employed in oncology, where doses tend to be increased up to or near the maximally tolerated levels.

There is still much debate on whether or not trials of combinations of therapies are warranted at this stage. Some have suggested trials of two and three agent combinations (such as AZT plus IFN-α plus a receptor-blocking agent). Others feel that there are so many agents to be evaluated that efforts should continue to focus on the potential efficacy of new compounds or modified compounds. In any event, many new agents will be evaluated in conjunction with continuing ZDV administration. Clearly, immune-based therapy is a dynamic, interesting and very challenging medical and scientific area.

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High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates

(antiviral agents/affinity/AIDS/receptor/envelope)

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ABSTRACT **There is substantial evidence supporting the CD4 molecule as the principal cellular receptor for the human immunodeficiency virus type 1 (HIV-1). A number of truncated recombinant soluble CD4 (sCD4) molecules have been produced and shown to easily neutralize infection of laboratory strains of HIV-1 *in vitro*, and clinical trials using these sCD4 preparations have begun in patients with AIDS. Infectious HIV-1 titers in the plasma and peripheral blood mononuclear cells of five patients receiving sCD4 at 30 mg/day were sequentially monitored. No significant decrease in viral titers was found during therapy. Furthermore, plasma samples from eight patients with AIDS were titrated for HIV-1 with and without the addition of sCD4 *ex vivo*. Despite the addition of sCD4 at up to 1 mg/ml, there was little change in plasma viral titers. Subsequently, 10 primary HIV-1 isolates were tested for their susceptibility to neutralization *in vitro* by one preparation of sCD4. Neutralization of these clinical isolates required 200–2700 times more sCD4 than was needed to inhibit laboratory strains of HIV-1. Similar results were observed using one other monomeric sCD4 preparation and two multimeric CD4–immunoglobulin hybrid molecules. We conclude that unlike laboratory strains, primary HIV-1 isolates require high concentrations of sCD4 for neutralization. This phenomenon may pose a formidable problem for sCD4-based therapeutics in the treatment of HIV-1 infection.**

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS (1). There is strong evidence showing that the CD4 molecule on helper T cells and monocyte/macrophages is the principal receptor for HIV-1 (2, 3). Consequently, attempts have been made to create a soluble form of CD4 (sCD4) that would block HIV-1 binding and penetration. CD4 has been cloned from the T4 lymphocyte and expressed in large quantities as truncated recombinant sCD4 (4, 5). *In vitro* experiments have demonstrated significant neutralization of HIV-1 infection in T-cell lines with sCD4 at ≈100 ng/ml. These studies used HIV-1 isolates that had been propagated in the laboratory for many years. In addition, sCD4 blocked *in vitro* infection by the simian immunodeficiency viruses and the human immunodeficiency virus type 2 (HIV-2) (6), although inhibition of HIV-2 required 25 times higher concentrations of sCD4 than the tested laboratory strains of HIV-1 (7). Furthermore, several investigators have developed constructs that link sCD4 to IgG, IgM, or a cytotoxin and have shown their effectiveness *in vitro* against laboratory strains of HIV-1 [8–11].

Phase I/II clinical trials using sCD4 have been initiated based on its potent antiviral activity *in vitro* against laboratory isolates of HIV-1. We now report the lack of detectable anti-HIV-1 effect *in vivo*, as determined by quantitative viral cultures, in one phase I/II clinical trial of sCD4 despite seemingly adequate blood levels. In addition, extensive characterization of the effect of several sCD4 preparations on HIV-1 *ex vivo* and *in vitro* has subsequently revealed that much higher concentrations of sCD4 are required to neutralize unselected or minimally selected (through *in vitro* cultivation) primary viral isolates.

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METHODS

Quantitation of HIV-1 *in Vivo* in the Phase I/II Clinical Trial of sCD4. The study protocol as well as the clinical and laboratory results of the phase I/II trial of sCD4 (Biogen) (5) in patients with advanced AIDS-related complex or AIDS have been described (12). In addition to measuring serum p24 core antigen levels as a marker of viral burden, an end-point-dilution culture method (13) was used for serial quantitation of HIV-1 in the peripheral blood mononuclear cells (PBMCs) and plasma of five patients treated at our center with sCD4 at 30 mg/day, intramuscularly.

Quantitation of HIV-1 in Patient Plasma After the Addition of sCD4 *ex Vivo*. Portions of cell-free plasma samples from eight AIDS patients were mixed with sCD4 (Biogen) at 0, 0.1, 1, 10, 100, and 1000 μg/ml; except in one plasma sample (patient K) where the maximum sCD4 concentration was 10 μg/ml. Subsequently, the plasma/sCD4 mixtures (at 1000, 100, 40, 10, 1, and 0.1 μl) were titrated by end-point-dilution cultures (13) to determine if there were changes in the plasma HIV-1 titers as the concentration of sCD4 increased.

HIV Isolates and Their Titrations. Primary HIV-1 isolates for *in vitro* experiments were obtained as described (13). These clinical isolates were cultured once in PBMCs from the plasma of two patients with acute HIV-1 infection (patients P and Q), an asymptomatic seropositive person (patient R), and four patients with AIDS (patients E, K, O, and S). In addition, two distinct primary viral isolates, HIV(JR-FL) and HIV(JR-CSF) (14), were kindly provided by I. Chen (UCLA School of Medicine, Los Angeles). An infectious molecular clone was obtained from a short-term culture of HIV(JR-CSF), which, when transfected into human cells, produced progeny viruses that were lymphotropic but weakly monotropic (14). In contrast, the molecularly cloned HIV(JR-FL) isolate was found to be both lymphotropic and monotropic. Finally, we isolated HIV-1AC from a patient with Kaposi sarcoma (15). This isolate was obtained from plasma after a single short-term culture using PBMCs. HIV-1AC was also propagated in H9 cells for ≈1 year. Both the early and late isolates were employed in the *in vitro* studies. Laboratory HIV isolates tested included strains HTLV-IIIB and HTLV-IIIRF and the HIV-2 isolate LAV-2_{ROD}, all of which have

Abbreviations: HIV-1 and HIV-2, human immunodeficiency virus type 1 and type 2, respectively; sCD4, soluble CD4; PBMC, peripheral blood mononuclear cell; TCID, infectious dose in tissue culture; $TCID_{50}$, median infectious dose in tissue culture; P1, passage 1; ID_{90}, 90% inhibitory dose.

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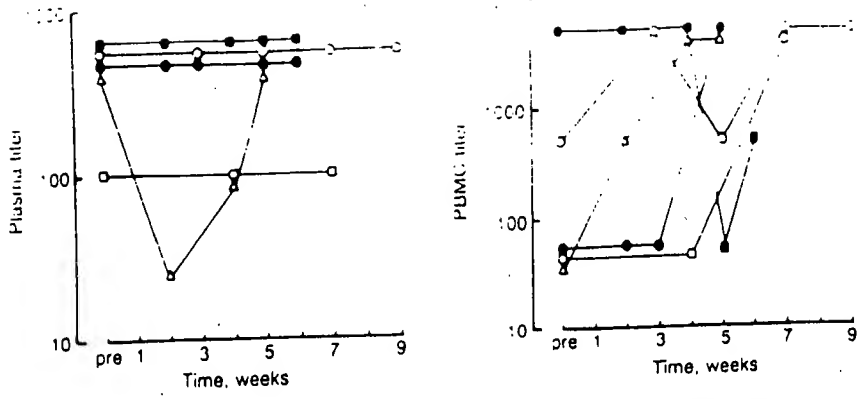

FIG. 1. Sequential HIV-1 titers in plasma (TCID per ml) (*A*) and PBMCs (TCID per 10^6 cells) (*B*) of five patients receiving sCD4. Patients: ■, A; ○, B; □, C; △, D; ●, E.

been cultured in T-cell lines for several years. These laboratory isolates were subsequently cultured and titrated in PBMCs by the end-point-dilution method prior to use in neutralization experiments *in vitro*.

Neutralization of Primary and Laboratory Isolates of HIV *in Vitro* with sCD4 or Leu3A. Neutralization assays were performed as we have described (16) using an inoculum of 50 $TCID_{50}$ (median infectious dose in tissue culture) incubated with 0, 0.1, 1, 10, 100, and 1000 μg of sCD4 (Biogen) (5) for 30 min at 37°C. On day 7, the p24 antigen levels in the sCD4-treated cultures were compared with values from an untreated culture, and results were then expressed as percent neutralization relative to the control. Similar neutralization studies were performed on a limited number of HIV-1 isolates using another monomeric sCD4 molecule (Smith Kline Beecham) (4) and the sCD4 hybrid constructs IgG–T4 and IgM–T4 provided by A. Traunecker (Bazel Institute, Switzerland) (8). IgG–T4 (90 kDa) is a hybrid molecule linking two sCD4 molecules to a portion of IgG, and IgM–T4 (700 kDa) has 10 valencies of sCD4 plus a portion of IgM. Finally, experiments to block infection by HIV-1 were also done on PBMCs and monocyte/macrophages by incubating the target cells for 30 min with Leu3A (Becton Dickinson), an anti-CD4 monoclonal antibody known to block HIV-1 infection (2, 3), prior to the addition of the viral inoculum.

RESULTS

HIV-1 Titers in Patients Receiving sCD4. End-point-dilution cultures were set up serially with the plasma and PBMCs of five patients treated with sCD4 at 30 mg/day. These patients were on therapy daily for 5–9 weeks with a mean steady-state serum sCD4 level of 156 ng/ml (12). The results of their sequential HIV-1 titers in plasma are summarized in Fig. 1*A*. Four patients had a pretherapy plasma titer of 500 infectious doses in tissue culture (TCID) per ml, and only patient D showed a transient decrease in titer to 25 TCID per ml on week 2, which returned to a level of 500 TCID per ml on week 5. HIV-1 titers in plasma samples from patients A, B, and E remained constant at 500 TCID per ml throughout the treatment period. Patient C had an initial plasma HIV-1 titer of 100 TCID per ml, which did not change during 7 weeks of treatment.

The pretherapy HIV-1 viral titers in PBMCs of these same patients ranged from 50 to 5000 TCID per 10^6 cells. Their serial PBMC titers while receiving sCD4 are summarized in Fig. 1*B*. Patient A had a pretherapy titer of 5000 TCID per 10^6 cells, which declined to 50 TCID per 10^6 cells on week 5 and subsequently increased to 500 TCID per 10^6 cells on week 6. Patients B, C, D, and E did not show decreases in HIV-1 titers in their PBMCs. In fact, patients (B, C, D, and E)

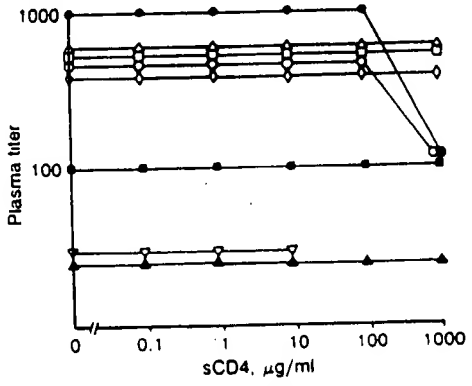

FIG. 2. HIV-1 titers in plasma (TCID per ml) after the addition of sCD4 *ex vivo*. Patients: △, A; ●, F; ○, G; □, H; ◇, I; ■, J; ▽, K; ▲, L.

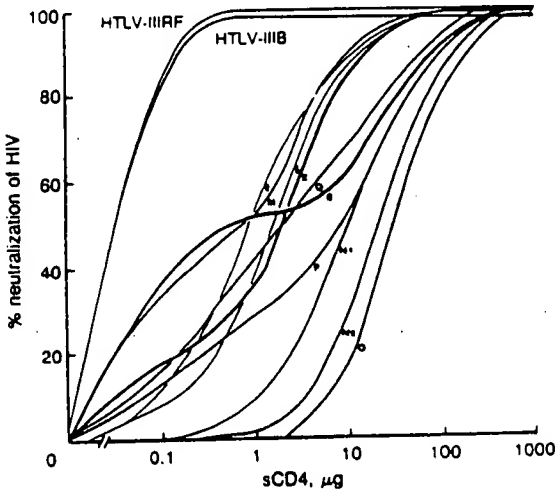

FIG. 3. HIV neutralization by sCD4 (Biogen) *in vitro*. The result for each isolate is represented by a computer-generated best-fit curve and is identified by a letter to its immediate right. Isolates: M, HIV-2 isolate LAV-2_{ROD}; N1, HIV(JR-FL); and N2, HIV(JR-CSF). Other primary HIV-1 isolates are identified by the letter codes used in Table 1.

Table 1. Summary of laboratory and primary [illegible] for four sCD4 molecules against each isolate

Patient	Isolate	Properties of viral stocks: TCID$_{50}$ per ml	p24, ng/ml	RT activity, cpm × 10^{-3}/ml	sCD4-Biogen	sCD4-S-K	[illegible]
	HTLV-IIIB	1 × 10^4	500	90.5	0.07	0.03	[illegible]
E	E	1 × 10^6	286	12.3	25.0	ND	ND
K	K	2 × 10^3	10.2	9.30	38.0	ND	ND
M	LAV-2$_{ROD}$	1 × 10^3	1.50	44.7	12.0	ND	ND
N1	HIV(JR-FL)	2 × 10^4	170	98.0	110	50	2.40
N2	HIV(JR-CSF)	1 × 10^5	97.0	25.0	180	ND	40.0
O	O	1 × 10^4	1.00	613	190	ND	ND
P	P	1 × 10^4	25.0	22.3	110	70	8.00
Q	Q	1 × 10^3	50.0	5.50	72.0	ND	ND
R	R	1 × 10^3	30.0	15.5	85.0	ND	ND
S	S	1 × 10^4	1.50	54.5	30.0	ND	ND
T1*	HIV-1AC	2 × 10^4	573	34.7	90.0	ND	ND
T2†	HIV-1AC	1 × 10^3	242	320	0.90	ND	ND

S-K, Smith Kline Beecham; RT, reverse transcriptase; ND, not done.
*P1.
†After 1 year of propagation in H9 cells.

demonstrated an increase in PBMC titers during therapy. We, therefore, conclude that sCD4 (as high as 30 mg/day) did not have a detectable anti-HIV-1 effect *in vivo*, as determined by serial viral titers in plasma and PBMCs.

HIV-1 Titers in Plasma After the Addition of sCD4 *ex Vivo*. Eight patients (A, F, G, H, I, J, K, and L) with AIDS had their plasma titrated for HIV-1 with and without the addition of sCD4 *ex vivo*. Fig. 2 summarizes the quantitative results of these *ex vivo* experiments and shows that the untreated plasma titers ranged from 25 to 1000 TCID per ml. The plasma samples from patients F and G were the only specimens to demonstrate a decrease in viral titer when mixed with sCD4; however, this required a concentration of 1 mg/ml. HIV-1 titers in other plasma samples (from patients A, H, I, J, and L) were unaffected by sCD4 at 1 mg/ml, whereas the titer was unchanged in the plasma of patient K with sCD4 at 10 μg/ml. Even those specimens with low pretherapy plasma titers of 25 TCID per ml (from patients K and L) were not affected by the addition of sCD4. Overall, it appears that unselected primary HIV-1 isolates in plasma are extremely resistant to neutralization by sCD4.

Neutralization of Primary and Laboratory Isolates by sCD4 *in Vitro*. Experiments were performed to compare sCD4 neutralization of the infectivity of primary and laboratory (HTLV-IIIB, HTLV-IIIRF, and LAV-2$_{ROD}$) strains of HIV in stimulated PBMCs from normal donors. Primary viral isolates were cultured only once in PBMCs and thus referred to as passage one (P1) viruses. The results of these neutralization experiments, summarized in Fig. 3, show that, when compared to HTLV-IIIB and HTLV-IIIRF, there is a marked decrease in the effectiveness of sCD4 (Biogen) to neutralize infection by all P1 isolates as well as LAV-2$_{ROD}$. The 90% inhibitory dose (ID$_{90}$) of sCD4 for HTLV-IIIB and LAV-2$_{ROD}$ was 0.07 μg and 12 μg, respectively, whereas the ID$_{90}$ for primary isolates ranged from 15 to 190 μg (Table 1). The ID$_{90}$ for the primary isolates was 200–2700 times higher than that of HTLV-IIIB.

Fig. 4*A* shows that similar neutralization results were obtained using the sCD4 preparation (4) from Smith Kline Beecham. The ID$_{90}$ of this sCD4 molecule for HTLV-IIIB was 0.03 μg, for HIV(JR-FL) was 50 μg, and for isolate P was 70 μg (Table 1). In addition, Fig. 4*B* shows the effectiveness of the IgG–T4 construct in neutralizing HTLV-IIIB and three P1 isolates *in vitro*. Again, the primary HIV-1 isolates were substantially more refractory to this molecule when compared with HTLV-IIIB. The ID$_{90}$ for IgG–T4 against HTLV-IIIB was 0.02 μg, against HIV(JR-FL) was 2.4 μg, HIV(JR-CSF) was 40.0 μg, and against isolate P was (Table 1). Similar studies using IgM–T4 showed that th for HTLV-IIIB was 0.11 μg, compared to 0.7 μg for HI FL), 6 μg for HIV(JR-CSF), 25 μg for isolate P, and for isolate E (Table 1). Therefore, the relative resista primary P1 isolates to neutralization is not unique sCD4 preparation.

Neutralization experiments using sCD4 *in vitro* we formed using the initial P1 isolate of HIV-1AC, as well virus after 1 year of propagation in H9 cells. A n difference was observed in the sensitivity of sCD4 n ization for these two viruses (Fig. 5). The sCD4 ID$_{90}$ P1 isolate was 90 μg but was only 0.9 μg for the passaged in the laboratory (Table 1). It thus appears t least in this case, propagation of HIV-1 *in vitro* selec viral isolates that are more susceptible to neutralizat sCD4.

Effect of Target-Cell Differences on sCD4 Neutralizi tivity *in Vitro*. Several experiments were carried determine the effectiveness of sCD4 in neutralizing infection of different target cells. The results summari Fig. 6*A* demonstrated that sCD4 neutralizes HTLV-III HTLV-IIIRF with equal efficiency in PBMCs and H9 HIV(JR-FL) and isolate P were employed to exami neutralizing activity of sCD4 in PBMCs and mon macrophages, since both of these isolates replicate t

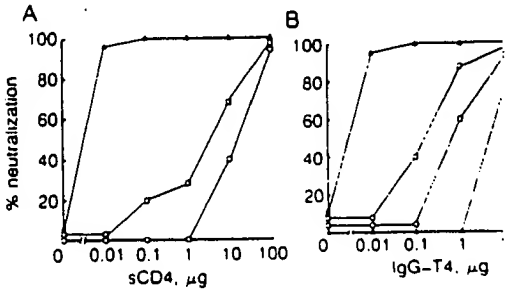

FIG. 4. Neutralization of HIV-1 by monomeric sCD4 fro Kline Beecham (*A*) and the multimeric IgG–T4 hybrid mole *in vitro*. Isolates: △, HTLV-IIIB; □, HIV (JR-FL); ▲, HIV(JI ○, P. All isolates were 100% neutralized at 100 μg, although points were displayed separated one from another for the clarity.

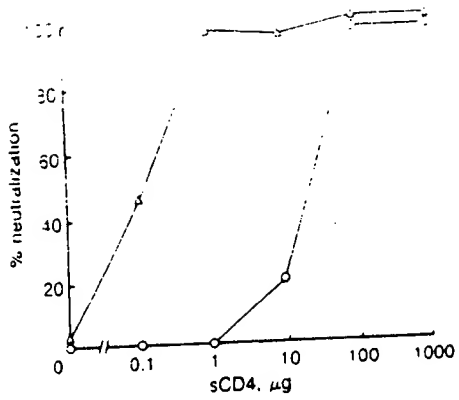

FIG. 5. Neutralization of the primary isolate HIV-1AC after a single passage in PBMCs (○) and after propagation in H9 cells for approximately 1 year (△).

levels in monocyte/macrophages. As shown in Fig. 6*B*, there was not a marked difference in sCD4 activity in these two types of target cells.

Exclusion of Two Possible Explanations for the Relative Resistance of Primary HIV-1 Isolates to sCD4. One explanation for the relative refractoriness of primary HIV-1 isolates to sCD4 was the possibility that these viruses may utilize an alternate CD4-independent mechanism to enter PBMCs. However, as shown in Fig. 6*C*, pretreatment of PBMCs with the anti-CD4 monoclonal antibody Leu3A resulted in dose-dependent inhibition of infection by HTLV-IIIB, HIV(JR-FL), and isolates P and Q. Complete inhibition was achieved with Leu3A at 1 μg/ml for these isolates. In addition, Leu3A also effectively blocked infection of monocyte/macrophages by two monotropic HIV-1 isolates [HIV(JR-FL) and P]. Therefore, the data do not support an alternate mechanism of entry for primary HIV-1 isolates.

Another explanation was the possibility that there may be excessive defective viral particles in the stock preparations of primary HIV-1 isolates. We, therefore, measured the p24 antigen concentration and the particulate reverse transcriptase activity in each virus preparation (Table 1) and then correlated these findings with the infectious titer ($TCID_{50}$ per ml) in each viral stock, as well as the ID_{90} of sCD4 against each HIV-1 isolate. Such analyses of the data outlined in Table 1 showed no evidence of excessive defective particles in the primary HIV-1 preparations (data not shown).

We have found no consistent decrease in plasma and PBMCs of five patients receiving sCD4 mg/day) for 5–9 weeks despite seemingly adequate serum levels (Fig. 1). Moreover, the addition of sCD4 (to 2 mg/ml) *ex vivo* to plasma samples from eight AIDS patients did not significantly lower their plasma HIV-1 titers, even in the two samples with relatively low viral burden (25 TCID per ml) (Fig. 2). These findings were unexpected given that previous studies have shown potent neutralizing activity of sCD4 against laboratory strains of HIV-1 *in vitro* (4–6). Therefore, several experiments were performed to address this apparent discrepancy. First, the lack of a detectable anti-HIV-1 effect of sCD4 in experiments represented by Figs. 1 and 2 could be due to an interfering factor present in plasma, such as a blocking antibody (17). The addition of heat-inactivated subneutralizing amounts of seropositive plasma to sCD4, however, did not affect its potent neutralizing activity against HTLV-IIIB (data not shown), suggesting that, at least at the amounts tested, plasma interference is not an explanation, as has been shown by others (18). This conclusion is supported by the observation that primary P1 isolates are quite resistant to sCD4 even in the absence of plasma (see below). A second possible explanation for the discrepancy is the difference in target cells given that prior studies of sCD4 against laboratory isolates of HIV-1 were performed in T-cell lines, whereas our studies were carried out in stimulated PBMCs from normal donors. The results in Fig. 6 *A* and *B*, nevertheless, did not show any significant differences in sCD4 activity using several types of target cells (H9 cells, PBMCs, and monocyte/macrophages). Lastly, the discrepancy could be due to intrinsic differences between laboratory strains and primary isolates of HIV-1. This explanation is strongly supported by the results in Figs. 3 and 5, showing that 10 primary P1 isolates were 200–2700 times more refractory to neutralization by one preparation of sCD4. Furthermore, this relative resistance of primary HIV-1 was also encountered with one other sCD4 preparation and two sCD4–immunoglobulin hybrid molecules (Fig. 4).

Although the reason for the refractoriness of primary HIV-1 isolates to sCD4 is unknown, it does not appear to be due to excessive defective interfering particles in the viral preparations. Our analyses of the data in Table 1 showed that there was no evidence of higher viral protein (p24 and reverse transcriptase) content per infectious unit in the primary HIV-1 stocks. In addition, an excess of interfering particles is unlikely to account for the relative refractoriness of

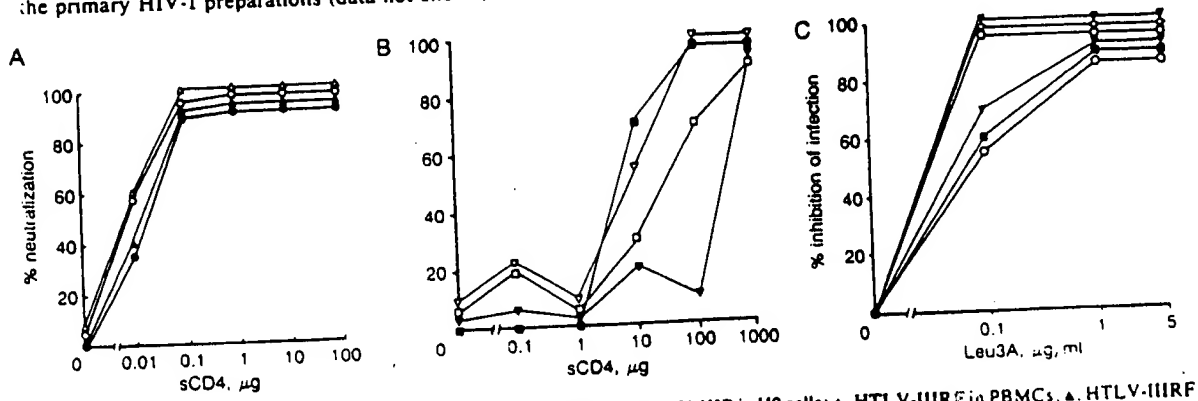

FIG. 6. (*A*) Neutralization of HIV-1 strains. ○, HTLV-IIIB in PBMCs; ●, HTLV-IIIB in H9 cells; △, HTLV-IIIRF in PBMCs. ▲, HTLV-IIIRF in H9 cells. All isolates were 100% neutralized at 0.1 μg. (*B*) Neutralization of primary isolates by sCD4 in PMBCs and monocyte/macrophages. ▽, Isolate P in PBMCs; ▼, isolate P in monocyte/macrophages; □, HIV(JR-FL) in PBMCs; ■, HIV (JR-FL) in monocyte/macrophages. (*C*) Neutralization of HIV-1 infection by pretreatment of target cells with the anti-CD4 monoclonal antibody Leu3A. ○, Isolate Q in PBMCs; the other symbols are defined in *A* and *B*. All viruses were 100% neutralized at 1.0 μg/ml.

HIV(JR-CSF) and HIV(JR-FL) to sCD4, because these viruses are produced from the transfection of infectious molecular clones, which is a situation not prone to generate defective particles. However, the determination of the free gp120 concentration in each of the viral stocks would be needed to completely exclude interference from defective particle formation. To address another possible explanation, results in Fig. 6*C* showed that infectivity of the primary isolates was completely blocked by an anti-CD4 monoclonal antibody (Leu3A at 1 μg/ml). Therefore, the refractoriness of primary HIV-1 isolates to sCD4 is also unlikely to be due to an alternate mechanism of viral entry into PBMCs. Regardless of the explanation, however, our findings do show conclusively that primary HIV-1 isolates are rather resistant to sCD4 neutralization *in vivo*, *ex vivo*, and *in vitro*.

We speculate that the lower efficacy of sCD4 observed in our studies is due to lower binding affinities between sCD4 and gp120 of primary HIV-1 isolates. Indeed, Moore (7) has demonstrated that the relative refractoriness of HIV-2 (LAV-2_{ROD}) to sCD4 is due to a 25 times lower binding affinity between its envelope glycoprotein and sCD4 compared with that of HTLV-IIIB gp120 and sCD4 (7). By analogy, we would estimate that the gp120–sCD4 affinities for our primary HIV-1 isolates may be 100–1000 times lower than 1.5–4.0 nM, which was previously reported for the HTLV-IIIB gp120–CD4 interaction (7).

In infected persons, HIV-1 is now known to be present as a population of related yet diverse viruses termed quasispecies (19). Upon *in vitro* cultivation, selection occurs and even a minor variant can become the predominant viral form if it has a growth advantage (19). Our results in Fig. 5 show that while the quasispecies in a primary P1 HIV-1 stock were rather resistant to sCD4, a later isolate obtained after 1 year of serial passages in H9 cells was relatively sensitive to sCD4 neutralization, similar to that seen with HTLV-IIIB and HTLV-IIIRF. This observation suggests that laboratory strains of HIV-1 represent one extreme of HIV-1 populations that has been selected perhaps because of a higher gp120–CD4 affinity and thus a growth advantage in $CD4^+$ cells. Indeed, a close examination of the results in Figs. 2 and 3, showing that unselected viruses in plasma appear to be even more resistant to sCD4 than primary P1 isolates, suggests that even a single short-term passage in PBMCs *in vitro* may have already selected out viral populations that are relatively more sensitive to sCD4.

Thus the overall findings presented here have several major implications. First, the relative refractoriness of primary HIV-1 isolates to sCD4 poses a formidable problem for sCD4-based therapeutics, including those second-generation products such as sCD4–immunoglobulin hybrid constructs (8, 9) or sCD4–toxin conjugates (10, 11). We expect that much higher doses of sCD4 or its use in combination with other antiviral drugs will be necessary to achieve a consistent anti-HIV-1 effect *in vivo*.

Furthermore, it should be determined whether the relative refractoriness of primary HIV-1 isolates to sCD4 is the consequence of lower gp120–CD4 binding affinities. If this were indeed the case, then the high affinity of gp120–CD4 binding previously reported for laboratory isolates would not be representative of the envelope–receptor interaction for most HIV-1 *in vivo*.

Another implication of our findings is that although it is convenient to test anti-HIV-1 drugs using laboratory viruses and tumor cell lines, any promising agent should also be examined against unselected HIV-1 *ex vivo* and minimally selected P1 isolates *in vitro*, using normal cells as targets for infection. Despite the urgent need to develop AIDS drugs quickly, careful preclinical studies that would closely resemble the *in vivo* situation should be conducted and must not be bypassed or short changed. Similarly, basic investigations on the biology of HIV-1 should include studies on primary viral isolates that have not been highly selected under artificial conditions.

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Toward an Understanding of the Correlates of Protective Immunity to HIV Infection

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Considerable progress has been made recently in understanding the genetic, immunologic, and virologic factors in human immunodeficiency virus (HIV)–infected individuals who either rapidly progress or do not progress to acquired immunodeficiency syndrome (AIDS). In addition, detection of HIV-specific immune responses in HIV-negative individuals who have been exposed to the virus multiple times suggests that natural immune responses to HIV may be protective in rare individuals. Understanding the correlates of protective immunity to HIV infection is critical to efforts to develop preventive HIV vaccines as well as to determine the feasibility of treating HIV infection by boosting immunity to HIV.

A spectrum of clinical courses can occur after HIV infection. Approximately 10% of HIV-infected subjects progress to AIDS within the first 2 to 3 years of HIV infection (rapid progressors) (*1*, *2*); approximately 5 to 10% of HIV-infected subjects are clinically asymptomatic after 7 to 10 years and have stable peripheral blood $CD4^+$ T cell levels (nonprogressors) (*1*, *2*); and the remaining HIV-infected subjects are projected to develop AIDS within a median time of approximately 10 years from initial infection (typical progressors). Data from the Multicenter AIDS Cohort Study suggest that 20 years after infection, 10 to 17% of HIV-infected individuals will be AIDS-free (*1*, *2*).

In this article, we consider recent progress in understanding immunologic and virologic characteristics of HIV-infected typical progressors, rapid progressors, and nonprogressors; summarize data on host genetic factors that may determine the effectiveness of immune responses to HIV; and summarize goals of future research.

Typical Progressors

In typical progressors, within weeks of HIV infection, viremia falls coincident with the induction of anti-HIV cellular and humoral immune responses (3). The fall in viremia correlates best with the appearance in peripheral blood of anti-HIV major histocompatibility complex (MHC) class I–restricted $CD8^+$ cytotoxic T cells (CTLs) (3).

During acute HIV infection there is oligoclonal expansion of V_β immunoglobulin families, predominantly restricted to $CD8^+$ T lymphocytes; within this population are contained HIV-specific CTLs (4). Mobilization of a restricted T cell receptor–for–antigen repertoire may be ultimately associated with a less effective immune response, thus facilitating persistence of HIV (*4*, *5*).

$CD8^+$ T cells are thought to be important in the immune response to HIV during the latent phase of HIV infection for the elimination of productively infected cells and for control of the viral load (6). However, HIV-specific $CD8^+$ CTLs may also be involved in the immunopathogenesis of HIV infection; they may contribute to the depletion of antigen-presenting cells either through a direct mechanism (that is, killing of the virus-expressing antigen-presenting cells) or indirectly through tissue damage after the release from CTLs of certain cytokines such as tumor necrosis factor α/β and interferon γ during the process of cytolysis (*5*, *7–10*). Nowak *et al.* have hypothesized that patients whose immune systems recognize fewer immunodominant HIV epitopes have a more stable and effective immune response to HIV than those whose CTL responses are against multiple, less dominant epitopes (*11*).

In addition to CTLs, neutralizing antibodies may be a component of the initial control of HIV replication (*12*, *13*). However, as HIV variants emerge over time, new variants frequently are not neutralized by autologous sera, and in some cases, antibodies against newly emerging HIV variants may enhance HIV replication in vitro (*12*, *13*), although the significance in vivo of enhancing antibodies is controversial (*13*). Heath *et al.* have reported that HIV virions coated with neutralizing antibody and attached to tonsillar follicular dendritic cells were still infectious for $CD4^+$ T cells (*14*). This study raised the important question of whether neutralizing antibodies can prevent dendritic cell–associated HIV infectivity in vivo. A randomized trial of passive immunotherapy of HIV-infected patients suggested that the administration of heat-inactivated plasma from HIV-infected individuals every 2 weeks for 1 year could slow the progression to AIDS in the recipients (*15*). Thus, antibodies appear to be involved in protective immunity against the progression of HIV infection, although the specificities of anti-HIV neutralizing antibodies that might be protective in patients remain unresolved.

Progression to AIDS is associated with generalized activation of the immune system, manifested by elevated serum concentrations of neopterin, soluble interleukin-2 receptor, soluble CD8, and β_2-microglobulin, and with activation of a large proportion of $CD8^+$ T cells (*4*, *7*, *8*, *16*, *17*). HIV-infected cells, circulating virions, and viral particles trapped in the follicular dendritic cell network of lymph node and spleen maintain chronic stimulation of the immune system.

Several components of generalized immune activation associated with HIV infection, such as stimulation of different T cell subsets and high levels of antibody production with specificities against a large range of epitopes of different HIV proteins, reflect the efforts of the immune system to control the replication and spread of the virus. However, as the disease progresses, both cell-mediated and humoral immune responses are severely impaired, resulting at least in part from the loss of the regulatory function of $CD4^+$ T lymphocytes and defective or increased production of either immunoregulatory or proinflammatory cytokines or both (*18*). Thus, as a consequence of the impaired regulation of both T and B cell functions, immune activation may ultimately become inappropriate and detrimental effects will predominate.

Rapid Progressors

Rapid progressors have a rapid decline in $CD4^+$ peripheral blood T cell levels, usually within 2 to 3 years after primary HIV infection (*1*, *2*). In general, rapid progressors are characterized by lower levels of antibodies to HIV proteins (*1*, *2*, *19*, *20*) and by low or absent antibodies that neutralize autologous HIV variants (*19*, *21*). High levels of antibodies that enhance the growth of autologous HIV isolates in vitro have been reported in rapid progressors (*22*). Levy *et al.* have found that noncytolytic $CD8^+$ T cell responses that suppress HIV replication are initially present and then decrease in rapid progressors (*23*). Two groups (*24*, *25*) have recently reported identification of

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$CD8^+$ T cell soluble factors that inhibit HIV replication (*23*). Baier *et al.* report that the anti-HIV $CD8^+$ T cell factor is IL-16 (*24*), whereas Cocchi and colleagues showed that $CD8^+$ cell–derived chemokines RANTES, MIP-1α, and MIP-1β synergized to suppress HIV replication (*25*). It will be critical to determine whether production of any of these cytokines is defective in rapid progressors. Others have found anti-HIV $CD8^+$ CTL activity in rapid progressors (*4*, *26*, *27*). Rinaldo *et al.* reported low levels of memory $CD8^+$ CTLs by precursor frequency analysis in rapid progressors compared with nonprogressors, although anti-HIV CTL effector cell activity was present in fresh peripheral blood cells from rapid progressors that was comparable with CTL activity in nonprogressors (*26*). Other characteristics of rapid progressors include elevated numbers of activated $CD8^+$ $CD38^+$ DR^+ T cells (*27*) and elevated serum markers of immune activation (*1*, *2*, *28*).

A uniform finding has been a high viral load in rapid progressors that does not fall dramatically after primary HIV infection (Table 1) (*28–32*). But an issue that has yet to be resolved is the amount of heterogeneity of the viral load. Both Delwart *et al.* and Wolinsky *et al.* found more homogeneity in HIV isolates in rapid progressors compared with typical progressors and nonprogressors, implying that the immune response to HIV is ineffective in rapid progressors and is incapable of driving HIV variant diversification (*33*). In contrast, Yu *et al.* found more viral heterogeneity in HIV isolates over time in rapid progressors (*34*).

Nonprogressors

Nonprogressors have high levels of $CD8^+$ $CD38^-$ CTLs (*23*), high peripheral blood $CD8^+$ MHC class I–restricted anti-HIV CTL levels that do not fall over time (*19*, *35*), strong $CD8^+$ non-MHC–restricted HIV suppressor activity (*36*, *37*), and high levels of antibodies to HIV (*19*, *35*, *38*). Several investigators have reported increased neutralizing antibodies to HIV or a wide breadth of cross-reactive neutralizing antibodies (or both) in nonprogressors (*19*, *21*, *35*, *39*). Thus, neutralizing antibody levels may well be important for the control of HIV in nonprogressors, although the specificity of such salutary neutralizing antibodies is not known. Stable $CD4^+$ peripheral blood T cell levels are a hallmark of this group with low concentrations of serum and cellular markers of immune activation (*1*,*2*, *27*, *35*). Finally, in nonprogressors the structure and function of lymph node germinal centers are maintained and the follicular dendritic cells are preserved (*7*, *8*, *19*).

It has been proposed that cytolysis of HIV-infected antigen-presenting cells leads to early and severe immunosuppression and is crucial to AIDS pathogenesis (*40*). Zinkernagel and Hengartner have argued that in spite of in vitro cytolytic effects of HIV on susceptible T cell lines, HIV in vivo may not be a cytolytic virus, but rather induces profound $CD8^+$ T cell–dependent destruction of HIV-infected antigen-presenting cells and T cells (*41*). This hypothesis predicts that rare individuals will be able to eliminate HIV-infected cells with potent HIV-specific $CD8^+$ T cells (*41*). Similarly, if AIDS is primarily mediated through pathogenic CTL immune responses to HIV, then in this scenario an asymptomatic carrier state should exist in which there are high viral loads and essentially no anti-HIV $CD8^+$ T cell responses (*41*). The fact that recent data have demonstrated that the viral load is low and that anti-HIV CTL levels are generally high in nonprogressors argues against this latter hypothesis.

In contrast to rapid progressors, HIV variants in nonprogressors have been reported by some investigators to be diverse, suggesting that HIV variant heterogeneity may be a reflection of effective immune responses to HIV (*33*). Thus, in nonprogressors, it appears that immune responses are sufficently effective to maintain or at least markedly prolong the clinically latent phase of HIV infection.

There is also evidence that some nonprogressors are infected with constitutively less pathogenic or nonpathogenic HIV strains (Table 1). Thus, nonprogressors likely represent a heterogeneous group in whom host responses and the level of pathogenicity of the virus variably contribute to the state of nonprogression of HIV infection.

Multiply Exposed, HIV-Seronegative Individuals

Studies of individuals who have been exposed multiple times to HIV and are persistently HIV-seronegative have raised the possibility that, although these individuals show T cell responses to HIV proteins, a small percentage of them may be resistant to HIV, or may have been able to clear the infection without making antibodies to HIV (*37*, *42*, *43*).

Clues to the explanation of multiply exposed HIV-negative individuals comes from observations in rhesus macaques and chimpanzees of resistance to low doses of HIV or simian immunodeficiency virus (SIV) given intrarectally or intravaginally (*44*). Primate studies have suggested that there may be local cellular mucosal immune responses capable of protecting against low-dose mucosal HIV or SIV challenges (*44*). However, given the rapidity with which anti-HIV circulating CTLs arise in primary HIV infection of humans and yet do not usually prevent the development of AIDS (*3*, *4*), and the fact that anti-HIV CTLs develop in vertically infected children without usually protecting against progression of HIV infection (*45*), complete clearance of HIV infection by HIV-specific CTLs (if it occurs at all) must be a rare event (*45*). The timing and regional location of the appearance of CTLs may be important. If CTLs develop after the initial dissemination of the virus, they may not be capable of curtailing the progression of disease, whereas if CTLs are present at the site of challenge, that is, the genital mucosa before virus dissemination as in the case of preimmunization, adequate control of infection may be achieved. Arguing against this latter point is the fact that SIVgag-immunized rhesus monkeys with no antibodies to SIV but with high levels of anti-SIVgag CTLs were not protected when challenged with intravenous SIVmnc in vivo (*46*). However, the intra-

Table 1. Characteristics of HIV in typical progressors, rapid progressors, and nonprogressors to AIDS.

Clinical course	Comments
Typical progressors	Monocytotopic homogeneous HIV strains are transmitted during primary infection (*58*).
	HIV isolates during the clinically latent stage are initially monocytotopic, nonsyncytium-inducing, slowly replicating variants (*29*).
	HIV isolates during progression to AIDS are frequently more rapidly replicating, T cell–tropic variants (*6*, *29*, *58*).
Rapid progressors	High viral load in primary HIV infection that generally does not fall dramatically to the levels seen with typical progressors (*28–32*).
	Rapid progressors have higher levels of unspliced HIV mRNA compared to nonprogressors or typical progressors (*31*, *59*).
	Some rapid progressors may be infected with more rapidly replicating, virulent HIV strains (*29*, *30*).
Nonprogressors	Viral load is generally lower in nonprogresors than in rapid progressors (*19*, *29*, *31*, *32*, *35*).
	Some, but not all, nonprogressors may be infected with constitutively less pathogenic HIV variants (*60*, *61*).

venous nature of the challenge might have overcome any protection afforded by CTLs, whereas it is possible that these monkeys may have withstood a mucosal challenge.

Genetic Factors Implicated in Modulating Host Immune Responses to HIV Infection

The MHC class I and class II genes play a major role in determining the specificity of T and B cell antiviral immune responses. A number of MHC alleles as well as other host genetic factors have been described that may influence predisposition or protection against HIV infection or disease (Table 2).

There are several mechanisms whereby MHC-encoded molecules might predispose an individual to rapid or nonprogression to AIDS. First, having a certain MHC class I or class II allele could protect against HIV progression by serving as a restricting element for one or several immunodominant HIV T helper or CTL epitopes, thus promoting a salutary immune response to HIV and protection from progression to AIDS. Such a protective effect of the MHC class II E_{α}^{d} gene in the development of murine AIDS has been documented (*47*). Similarly, the lack of protective MHC alleles could predispose to developing AIDS because of a lack of salutary responses to HIV (*48*).

Second, having a certain MHC class I or class II allele could predispose an individual to pathogenic immune responses against a viral epitope in certain tissues such as the central nervous system or lungs, or against certain HIV-infected cell types such as monocyte (or macrophage) and dendritic cells. Similarly, the lack of an AIDS-promoting MHC allele would protect against pathogenic immune responses to HIV.

Third, having rare MHC class I and class II alleles could facilitate the rapid recognition of HIV-infected allogeneic cells during the early stages of HIV infection, thus promoting rejection of HIV-infected cells by means of alloreactive T cell responses (*49*). Sheppard and colleagues have shown that human sera from alloimmunized individuals neutralized HIV in vitro (*50*). Similarly, having common MHC alleles could promote less effective anti-HIV alloantigen responses and thus promote HIV infection or progression.

Fourth, human leukocyte antigen (HLA)–HIV disease associations are not absolute; thus, the data in Table 2 might reflect the association of genes linked to or within the MHC (*51*). For example, genetic markers linked to the *HLA-A1*, *CW7*, *B8*, and *DR3* haplotype, such as the complement C4 null allele (*C4AQO*) and a polymorphism in the tumor necrosis factor α promoter, have been suggested as MHC-linked gene candidates that might participate in a multigene effect on outcomes of HIV infection (*51*).

Fifth, the recent discovery that the level of MHC class I expression on virus-infected cells regulates the susceptibility of these cells to natural killer cell–mediated lysis provides a new area of investigation into the role of host MHC I genes in regulating the effectiveness of natural killer cell responses to HIV (*52*).

Finally, roles for transporter-associated with antigen-presenting (*TAP*) gene alleles have been proposed in determining the outcome after HIV infection (*48*, *53*). Data have suggested that combinations of MHC-encoded *TAP* and class I genes may synergize either in providing certain salutary anti-HIV responses or in avoiding pathogenic anti-HIV immune responses, or both.

Table 2. Genetic factors implicated in modulating host immune responses to HIV infection.

Factor	Effect	Reference
	Major histocompatibility loci-encoded genes	
B35, C4, DR1, DQ1	Associated with Kaposi's sarcoma	(*62*)
DR1	Associated with Kaposi's sarcoma	(*51, 63*)
DR2, DR5	Associated with Kaposi's sarcoma	(*63*)
DR5	Associated with Kaposi's sarcoma	(*63*)
Aw23, Bw49	Associated with Kaposi's sarcoma	(*63*)
B62	Associated with fever, skin rash in primary HIV infection	(*51*)
Aw19	Associated with HIV seropositivity in HIV multiply exposed individuals	(*49*)
A1, A24, C7, B8, DR3	Associated with rapid progression to AIDS	(*51, 64*)
*DR4, DQB1*0302*	Associated with rapid progression to AIDS	(*65*)
DR3, DQ1	Associated with rapid progression to AIDS	(*66*)
B35	Associated with rapid progression to AIDS	(*51, 67*)
TAP2.1	Promotes HIV progression to AIDS	(*48*)
DR5	Associated with thrombocytopenia and lymphadenopathy in HIV infection	(*68*)
DR5, DR6	Association of diffuse infiltrative $CD8^+$ lymphocytosis with Sjogren's-like syndrome in HIV	(*69*)
Bw4	Associated with slow decline in $CD4^+$ cell numbers	(*65*)
*B13, B27, B51, B57, DQB1*0302,0303*	Protects from progression to AIDS	(*48*)
A26, B38, TAP1.4, TAP2.3	Associated with ability to clear HIV infection in transiently infected HIV-seronegative individuals	(*53*)
A28, Bw70, Aw69, B18	Associated with protection from HIV infection	(*49*)
A32, B4, C2	Associated with long-term survival in HIV infection	(*70*)
*A11, A32, B13, C2, DQA1*0301, DQB1*0302, DRB1*0400, DRB4*0101*	Associated with long-term survival in HIV infection	(*70*)
	Other genes	
p53 tumor suppressor gene	Controls HIV replicative patterns and determinant of viral latency	(*71*)
Unknown inherited trait	Associated with PB mononuclear cell resistance to HIV infection in vitro	(*72*)

Summary and Future Directions

A pattern is emerging that many nonprogressors to AIDS have an immune response to HIV that is quantitatively and qualitatively superior to anti-HIV immune responses that occur in HIV-infected individuals who rapidly progress to AIDS. The HIV load in peripheral blood mononuclear cells varies widely from patient to patient and generally increases within individual patients as the disease progresses (*32*). Recent studies now suggest that the cellular viral load level is established early on in HIV infection and is a predictor of the subsequent clinical course, with smaller viral loads after serconversion predicting longer survival (*32*). The initial key unanswered question is whether a small viral load after seroconversion in nonprogressors is related to low pathogenecity of the infecting HIV strain, to a particularly effective anti-HIV immune response, or to both. The answer to this question may not be the same for all patients.

A second important issue that must be explored quickly is that of the role of the host genetic background in determining the rate of progression of the clinical course. Though HIV proteins are of sufficient size to contain many immunogenic epitopes, HIV proteins contain strongly immunodominant regions, as well as a myriad of regions with sequence similarities to a wide spectrum of host proteins. Thus, it is critical to determine if MHC-encoded or other host genetic factors are responsible for a qualitatively more effective anti-HIV immune response in nonprogressors. If, in fact, nonprogressors are genetically programmed to successfully

control HIV, then immune reconstitution to rebuild a "better immune system" with allogeneic bone marrow and thymus grafts becomes a theoretical possibility.

Third, the need for determining the specificity of both CTL responses and protective neutralizing antibodies in nonprogressors is extraordinarily important for understanding both the biological basis of the nonprogressor status and the design of effective HIV vaccine immunogens. It is critical to determine the specificity of serum antibodies in nonprogressors that neutralize HIV primary isolates grown in peripheral blood mononuclear cells.

Fourth, the role of viral factors in determining nonprogressor status must be better understood. Although recent data demonstrated that *nef*-deleted mutants are not a common finding in nonprogressors (*61*), some investigators have found that HIV is more difficult to isolate from nonprogressors compared with typical progressors (*19*). The key question is whether a particular virus type or strain interacts with a particular host genotype to eventuate in nonprogressor status.

Fifth, the types of anti-HIV immune responses that are generated by small- and large-inoculum HIV infection through genital mucosa are critical to understand. More sensitive and inexpensive assays of HIV viral load are needed to determine the levels of HIV infection in various tissues. Studies are needed that profile mucosal and systemic immune responses to HIV after both genital and systemic routes of HIV infection, and that determine what immune responses are protective for systemic and genital challenges in animal models such as SIV infection of rhesus macaques.

Sixth, although the use of attenuated HIV strains as a vaccine remains controversial, attenuated SIV strains have protected adult rhesus monkeys against SIV challenge (*54*), and primary infection with HIV-2 may confer some protection against HIV-1 in humans (*55*). It is important to understand the correlates of protective immunity in these settings. Of note is the fact that although live-attenuated *nef*-deleted SIV is not pathogenic in adult rhesus monkeys, it may be pathogenic in neonatal animals (*56*). The immune and other factors in neonatal and adult rhesus monkeys that lead to protection in adults and to disease in neonates are critical to understand.

Finally, the role of pathogenic compared with salutary $CD8^+$ T cell responses to HIV in determining the various clinical courses of HIV infection must be defined. The role of $CD8^+$ T cell cytokines in suppression of HIV replication in long-term nonprogressors is particularly important to study. The fact that nonprogressors have high levels of anti-HIV CTLs strongly suggests that CTL responses to HIV may be important in the control of virus replication over time. However, the question of whether qualitative differences among anti-HIV CTLs, including the possibility of pathogenic effects, has not yet been resolved. Resolving this question is critical to HIV vaccine immunogen design and to the design of novel strategies to induce protective immune responses in those patients early in the clinical course of progression to AIDS. It has been hypothesized that CTL immune responses may be more effective if they are targeted at a major immunodominant epitope of HIV rather than at several less dominant regions (*11*). If this were true, it is possible that successful immunotherapy of a HIV-infected patient might boost the CTL response to a single conserved epitope, making it immunodominant by increasing the frequency of reacting T cells and inducing a more stable and effective CTL response (*11*). Such a trial of peptide-based immunotherapy has just begun in HIV-infected patients (*57*).

We are clearly entering a new era of understanding the pathogenesis of HIV infection and of appreciation of the novelty and complexity of the cellular and molecular mechanisms of HIV-host interactions. This new knowledge has reinforced the conviction that to develop effective anti-HIV drugs and vaccines, viral pathogenesis and the correlates of protective immunity must be understood.

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Toward an Understanding of the Correlates of Protective Immunity to HIV Infection

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empirical data on ecological safety issues.

EPA officials, however, show no signs of moving in that direction. And USDA officials have recently moved toward simpler, instead of more stringent, regulatory procedures for evaluating proposals for field testing transgenic plants. USDA officials, in fact, argue that a streamlined regulatory approach will "cut costs, encourage biotechnology innovations, and focus agency regulatory resources on the areas of greatest complexity."

—Jeffrey L. Fox

No winners against AIDS

No therapy has proved itself against HIV, not a preventive vaccine nor a therapeutic vaccine nor any of the immune-system-boosting treatments.

WASHINGTON, D.C.—The quest to develop both preventive and therapeutic HIV vaccines is proving a frustrating enterprise. Equally frustrating are efforts to jump start the waning immune systems of HIV-infected individuals. Indeed, these AIDS-research frustrations were everywhere in evidence during the "First National Conference on Human Retroviruses and Related Infections" held here recently.

One theme that emerged at the conference, however, is that cytotoxic T lymphocytes (CTLs) appear to play a prominent role in maintaining the relative health of most HIV-infected individuals who do not progress to full-blown AIDS. According to Thomas Harrer at Massachusetts General Hospital (Boston, MA), HIV-infected individuals who nonetheless have remained healthy for periods of 10 years or more show a strong CTL response against HIV. Their immune systems, moreover, tend to recognize a variety of specific HIV epitopes. Some of these epitopes, which are being evaluated, could prove "attractive targets for immunotherapeutic interventions," Harrer concludes.

Yet these evaluations will likely be complicated because the CTL response is not to whole viruses, or even viral proteins, but to small viral peptides. These viral peptides, furthermore, are presented to the immune system in conjunction with the body's complex and still poorly understood major histocompatibility complex (MHC) molecules. Indeed, variations in individual MHC molecules may help explain why some individuals are more susceptible to the ravages of HIV than others.

Another prominent conference theme is that none of the numerous HIV vaccines have distinguished themselves. Among firms clinically testing HIV-envelope-protein vaccines are Chiron (Emeryville, CA), Genentech (S. San Francisco, CA), and MicroGeneSys (Meriden, CT). Immune Response (San Diego, CA), for its part, is testing a killed-virus vaccine. According to Deborah Birx of the Walter Reed Army Institute of Research (Rockville, MD), "in our hands, there are no significant differences among the envelope-protein vaccines." She adds, though, that preliminary data from phase II trials of these vaccines indicate that some individuals are showing improved CTL functions and other signs of beneficial immune-system responses.

However, results from *in vitro* assays evaluating the envelope-protein vaccines "give us pause," notes Mary Lou Clements of the Johns Hopkins School of Hygiene and Public Health (Baltimore, MD). Even though the vaccines stimulate the production of neutralizing antibodies in recipient individuals and even though these antibodies react with laboratory strains of HIV, the antibodies react "poorly, if at all, with fresh field isolates of HIV," says Clements. "This may be the fault of the assays. But we have a lot of work to do to clarify these results."

Regarding the therapeutic use of HIV vaccines, Robert Schooley of the University of Colorado Health Sciences Center (Denver, CO) says that "we can say that they can be given safely, but efficacy is far from demonstrated. And we can't say that any one is more promising than the others."

In addition to these HIV vaccines, Ronald Desrosiers of Harvard Medical School's primate facility (Southboro, MA) is working on a live HIV vaccine that is genetically modified so that it cannot replicate. However, Desrosiers says that there are many obstacles to developing such a vaccine. And Sten Vermund of the National Institute for Allergy and Infectious Diseases (NIAID, Bethesda, MD) agrees, stating that "there is no strong industry champion for a live, attenuated HIV vaccine." Yet the approach may be seen in a more favorable light if multideletion mutants prove as effective as the simpler single-deletion versions that have been tested so far, notes Vermund.

In fact, several live-virus vaccines based on viruses other than HIV—including vaccinia and cowpox—are being studied. They are being engineered to carry genes specifying one or more HIV polypeptides. Yet none among this group has emerged as a front runner.

Another theme explored at the conference involves efforts by researchers to boost the immune response in HIV-infected individuals through the administration of cytokines. For example, when interleukin-2 was intermittently administered to a small group of these individuals, more than half showed a "striking" improvement in immune functions, including increases in CD4 T lymphocytes, according to Anthony Fauci, director of the NIAID. "I have to underscore that, although these are the most dramatic increases of CD4 cells of any intervention, we must be circumspect, because we have no clinical correlates," Fauci says.

Another cytokine approach involves efforts to expand immune-system cells *ex vivo*, before reinfusing them into patients. One such approach is described as "adoptive cellular immunity" by Judith Lieberman of the New England Medical Center (Boston, MA). Lieberman extracts an HIV-infected individual's T lymphocytes, treats them *in vitro* with cytokines and HIV-derived peptides to boost CTL activity, and then reinfuses them into patients. "We have had encouraging preliminary results, including no acute toxicity and significant enhancement of HIV-peptide-specific cytotoxicity," says Lieberman. "Some patients had sustained increases in CD4 cell counts for many weeks after a single infusion."

Nonetheless, despite these positive results, AIDS researchers inevitably come back to the conference's central theme. No therapy has emerged as a sure winner in the campaign against HIV, not a preventive vaccine nor a therapeutic vaccine nor any of the immune-system-boosting treatments.

—Jeffrey L. Fox

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Intercellular adhesion molecule 3, a candidate human immunodeficiency virus type 1 co-receptor on lymphoid and monocytoid cells

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The CD4 molecule serves as the principal cell surface receptor common to both the human and simian immunodeficiency viruses (HIV-1, HIV-2 and SIV). Since binding to CD4 is not sufficient to permit virus entry, HIV 'co-receptors' have been implicated in mediating the fusion of viral and cellular membranes necessary for completing the entry process. In order to identify candidate co-receptor molecules, a panel of monoclonal antibodies (MAbs) directed against adhesion molecules was tested for the ability of the MAbs to inhibit HIV-1-induced cell fusion (syncytium formation) and HIV-1 entry. Certain antibodies directed against CD18, CD11b and CD11c inhibited HIV-1-induced syncytium formation but not entry, in agreement with previous reports. Interestingly, certain antibodies to ICAM-3 (intercellular adhesion molecule 3) (CD50) significantly inhibited HIV-1-specific entry but not syncytium formation using human SupT1 cells. Only one antibody directed against ICAM-3 significantly inhibited HIV-1-induced syncytium formation, entry and infectivity. Our results suggest that certain epitopes of ICAM-3 may be involved in mediating HIV-1-specific entry into lymphoid and monocytoid cells.

Introduction

A virus receptor is defined as an entity on the cell surface to which a virus specifically binds. A consequence of this interaction is internalization and entry into the susceptible host cell. The first retroviral receptor to be identified as a predefined cell surface molecule was that utilized by the human and simian immunodeficiency viruses (HIV-1, HIV-2 and SIV) (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Sattentau *et al.*, 1988). HIV, in common with the majority of other retroviruses studied, fuses cells following binding to CD4 by fusing the viral envelope and cellular plasma membrane at physiological pH (pH-independent entry) (Stein *et al.*, 1987; McClure *et al.*, 1988, 1990).

Confirmation that CD4 served as the principal receptor for HIV was achieved following transfection of the human CD4 gene into human HeLa cells. HeLa cells expressing human CD4 acquired susceptibility to infection (Maddon *et al.*, 1986). Interestingly, murine 3T3 cells transfected with human CD4 allowed only HIV binding but not the subsequent steps of membrane fusion culminating entry. This provided the first evidence that binding to CD4 alone was not sufficient and that the participation of other cell surface molecules, described as co-receptors or cofactors, was required to complete the entry process.

HIV co-receptors appear to be expressed on the majority of human and primate, but not rodent and non-primate cells (Tersmette *et al.*, 1989; Clapham *et al.*, 1991; Dragic *et al.*, 1992; Broder *et al.*, 1993; Harrington & Geballe, 1993). Despite utilizing CD4 as their principal receptor, HIV-1, HIV-2 and SIV exhibit subtle differences in tropism suggesting that they have the capacity to exploit distinct molecules as co-receptors (Clapham *et al.*, 1991; Dragic & Alizon, 1993).

In order to identify potential HIV-1 co-receptors, investigators have exploited the observation that HIV, in common with many retroviruses, has the ability to induce cell-to-cell fusion resulting in the formation of multinucleate giant cells termed syncytia (Fig. 1*a*) when infected and uninfected cells are mixed together (Dalgleish *et al.*, 1984; Braun *et al.*, 1994). Syncytium induction occurs as a result of interactions between viral envelope glycoproteins and virus-specific cell surface receptors since this effect can be inhibited using antibodies directed against virus receptors or neutralizing antibodies directed against viral envelope glycoproteins (Dalgleish *et al.*, 1984).

The leukocyte function antigen 1 (LFA-1, i.e. CD18/CD11a) has been implicated in HIV-1-induced syncytium formation and entry (Hildreth & Orentas, 1989; Goud-

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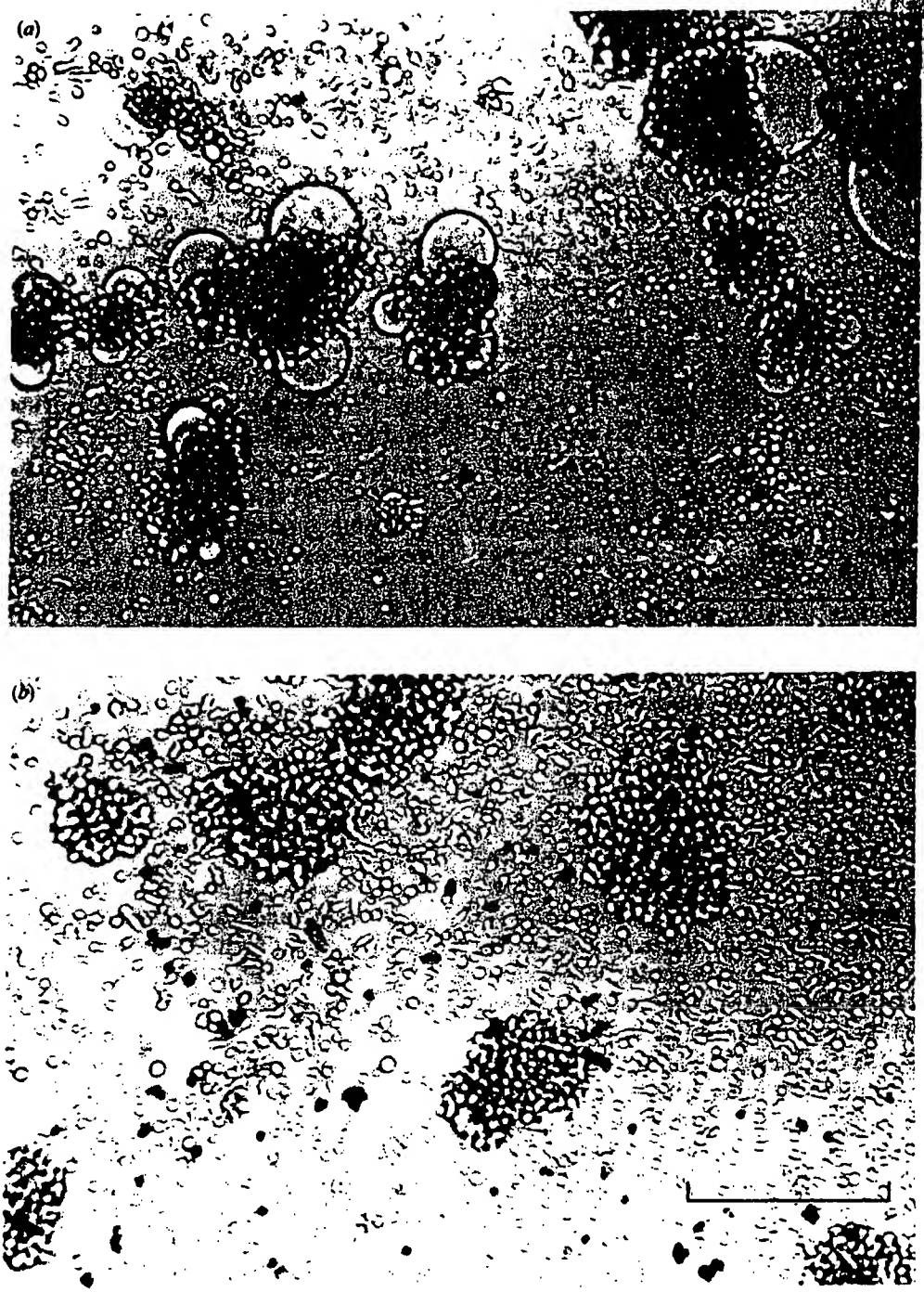

Fig. 1. (*a*) Illustration of syncytium induction resulting from co-cultivation of infected CEM/HIV_{Lai} cells with uninfected target SupT1 cells in the presence of an irrelevant antibody. The giant multinucleate syncytia are most notable as large transparent structures protruding from aggregated rather than isolated cells. (*b*) Illustration of inhibition of syncytium formation by an effective antibody (Table 2) or the control anti-CD4 antibody Q4120. These antibodies inhibited syncytium formation without dispersing the cell aggregates. Bar markers represent 400 μm.

smit & Śmit, 1990; Valentin *et al.*, 1990; Panteleo *et al.*, 1991). Since antibodies that inhibited syncytium formation did not inhibit HIV-1 entry, LFA-1 has been disregarded as a putative HIV co-receptor for mediating virus entry. However, the potential involvement of LFA-1 in the fusion of LFA-1-expressing cells remai (Panteleo *et al.*, 1991; Golding *et al.*, 1992).

In efforts to identify further candidate HIV c receptors, a panel of monoclonal antibodies (MAb directed against a variety of adhesion molecules w

for the ability of the MAbs to inhibit HIV-1-induced cell fusion. All the MAbs directed against a particular antigen for which some inhibition was observed were subsequently tested for their ability to inhibit HIV-1 entry. Entry was assayed using vesicular stomatitis virus (VSV) pseudotype viruses which represent a heterotypic virus mixture, where the core and genome are provided by the unrelated rhabdovirus VSV and the envelope glycoproteins are donated by HIV. These pseudotype viruses acquire the receptor host range and entry characteristics of the virus donating the envelope glycoproteins. The VSV pseudotype technique has been applied to a wide range of retroviruses including HIV (Sommerfelt & Weiss, 1990). It is particularly useful as a rapid and quantitative means to study virus receptors since it assays specifically virus binding and entry independently of later steps in the virus life cycle.

We have identified antibodies to cell surface molecules other than CD4 which nevertheless have significant effects on syncytium induction and/or HIV-1 entry. We present evidence suggesting that certain epitopes of the intercellular adhesion molecule 3 (ICAM-3), a molecule not previously implicated as an HIV co-receptor, may play a role in the entry of HIV-1 into lymphoid and monocytoid cells.

Methods

Viruses and cells. Human SupT1 and CEM cells (provided by Dr F. Barré-Sinoussi, Pasteur Institute, France) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), denoted 10% RPMI. Mink CCL64 (Henderson *et al.*, 1974) and human HeLa–CD4 cells (Akrigg *et al.*, 1991) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS.

The HIV_{Lai} laboratory-adapted strain of HIV-1 provided by Dr F. Barré-Sinoussi at a reverse transcriptase activity titre of 885000 c.p.m./ml was used to infect CEM cells. An equivalent of 9000 c.p.m. was used to infect 10×10^6 CEM cells in a volume of 200 µl 10% RPMI. After incubation for 1 h at 37 °C, the cells were washed twice and diluted to 1×10^6 cells/ml. The cells were incubated at 37 °C with 5% CO_2 and passaged at least twice before being used in the syncytial assays. The supernatant, harvested as a stock for infectivity assays, had a p24 value of 2 µg/ml and a $TCID_{50}$ value of 5×10^4 on SupT1 cells, and was stored at −135 °C.

Wild-type VSV Indiana strain was a gift from Dr Miklos Degré (University of Oslo, Norway).

Antibodies. A panel of 166 MAbs directed against adhesion molecules were provided by the Vth International Conference and Workshop on Human Leukocyte Differentiation Antigens as ascites fluids. A control purified antibody directed against human CD4, Q4120 (Healey *et al.*, 1990), was also provided by the workshop. All antibodies were initially tested at a final ascites dilution of 1:100 and a final concentration of 20 µg/ml for the control Q4120.

Syncytium inhibition assay. Syncytium inhibition assays were carried out as described by Clapham *et al.* (1991). Briefly, 50 µl SupT1 cells (at 10^6 cells/ml) were preincubated for 30 min at 37 °C with 50 µl of a 1:50 dilution of ascites fluid in 96-well trays (giving a final ascites dilution of 1:100) or 50 µl of control anti-CD4 antibody Q4120 giving a final concentration of 20 µg/ml. Then 50 µl CEM cells chronically infected with HIV_{Lai} were added (at 10^6 cells/ml). After overnight incubation syncytium induction was scored using light microscopy.

Pseudotype assay. Pseudotype viruses were generated by superinfecting chronically HIV-1-infected CEM cells with wild-type VSV at an m.o.i. of 10 p.f.u./cell in a cell density of 10^6 cells/0·2 ml serum-free medium. Following virus adsorption for 1 h at 37 °C, non-adsorbed VSV was washed away by centrifugation and the cells were finally resuspended in growth medium (10% RPMI) at a density of 2×10^6 cells/ml and incubated at 37 °C for 15 h. The supernatant was harvested, clarified by centrifugation (2000 r.p.m. for 10 min in a Beckman tabletop centrifuge) and stored at −135 °C. The fraction of virus corresponding to wild-type VSV was neutralized initially using a goat anti-VSV polyclonal antibody kindly provided by Dr R. A. Weiss (Institute of Cancer Research, London, UK) and later using a polyclonal anti-VSV antibody raised in rabbits (Animal Unit, Haukeland Hospital, Bergen, Norway). Non-neutralized VSV, corresponding to pseudotype viruses bearing HIV-1 envelope glycoproteins, denoted VSV(HIV) were titrated on SupT1 cells as described by Clapham *et al.* (1991). Briefly, neutralized VSV was plated onto 5×10^6 SupT1 cells immobilized onto poly-L-lysine-coated 6-well trays. Following adsorption of virus for 1 h at 37 °C, the inoculum was removed and the cells were overlaid with 10^6 mink cells (resistant to HIV-1) to enhance the visualization of plaques. When the mink cells had settled, the medium was removed and the cells were overlaid with 10% RPMI containing a final concentration of 0·6% Difco Bacto-Agar. The stock of VSV(HIV) used throughout this study had a titre of $2{\cdot}4 \times 10^4$ p.f.u./ml.

Inhibition assays using MAbs were carried out by preincubating the SupT1 cells with 200 µl ascites fluid at a dilution of 1:50 in serum-free medium before adding 200 µl pseudotype virus inoculum containing 100–150 p.f.u. (a countable number, just less than confluence).

Infectivity assay. SupT1 cells were seeded in a 96-well tray in a volume of 50 µl (10^6 cells/ml) and preincubated with a 1:50 dilution of ascites fluid for 30 min at 37 °C. Control anti-CD4 antibody was used at a final concentration of 20 µg/ml. SupT1 cells were infected with 25 $TCID_{50}$ units of the HIV_{Lai} stock (corresponding to 0·1 ng p24) for 2 h at 37 °C. The cells were then washed three times in serum-free medium and finally resuspended in 100 µl 10% RPMI containing a 1:100 final dilution of ascites fluid. Supernatant was harvested from the cells 6 days post-infection and the levels of extracellular p24 were monitored as described by Sundqvist *et al.* (1989).

Results

Syncytium inhibition assay

A total of 166 distinct MAbs were tested for their ability to inhibit HIV-1-induced cell fusion as described. Table 1 lists the antigens recognized by antibodies that failed to inhibit HIV-1-induced syncytium formation at a final ascites dilution of 1:100 where the control anti-CD4 antibody Q4120 was effective. Figure 1(*a*) represents syncytium formation resulting from overnight co-cultivation of CEM cells chronically infected with HIV_{Lai} and uninfected SupT1 target cells either in the absence of antibody or in the presence of an antibody that does not inhibit syncytium formation. Only one antibody to VLA-1 partially inhibited syncytium formation (50%

Table 1. *List of antigens for which the antibodies tested showed no inhibition of syncytium formation*

Antigen	No. of MAbs	Syncytium formation*	Antigen	No. of MAbs	Syncytium formation*
ICAM-2	3	++	VLA-5	7	++
VLA-3	2	++	CD29	7	++
CD54	11	++	CD49b	8	++
VCAM-1	2	++	CD49f	6	++
CD43	2	++	CD61	5	++
CD31	1	++	Beta[4]	3	++
CD44	2	++	CD51	4	++
HML-1	7	++	Beta[7]	2	++
CD41	1	++	CD56	1	++
CD49d	8	++	VLA-1	3	++ (+)†
Novel integrins and adhesion molecules				18	++
Controls					
None		++			
CD4	1	+/−			

* ++, Syncytium formation, no inhibition by antibody; +/−, reduction in syncytium formation by 80–90%.

† One antibody to VLA-1 partially inhibited syncytium formation (50% reduction).

reduction in syncytium formation), but none of the antibodies to VLA-1 had any effect on the entry of VSV(HIV) pseudotype viruses.

As shown in Table 2, significant inhibition of syncytium formation was observed with one antibody against CD18, one against CD11b, one against CD11c and one against CD50 (ICAM-3). Fig. 1(*b*) is representative of the inhibition of syncytium formation produced by these antibodies and the control against CD4. None of the antibodies that inhibited syncytium formation dispersed the cells preventing cell contact. This phenomenon has previously been reported for certain antibodies to CD18 not included in our panel (Valentin *et al.*, 1990).

Only one antibody (S121) out of a total of 17 directed against ICAM-3 (CD50) significantly inhibited HIV-1-induced cell fusion. Partial inhibition of syncytium formation was also noted at a 1:500 final dilution, showing that this antibody was less inhibitory than purified control antibody raised against CD4. To rule out the possibility that antibody S121 was reacting with CD4, we tested the ability of this antibody to inhibit HIV-1-induced cell fusion of HeLa cells that do not express ICAM-3. Despite being CD4+ lymphoid SupT1 cells, antibody S121 failed to inhibit syncytium induction with HeLa–CD4 cells. In fact, none of these antibodies to ICAM-3 could inhibit 2_{rod}-induced cell fusion of SupT1 cells which rules out the blocking of CD4 as a mechanism for the inhibition by antibody S121 (data not shown). Taken together, these results suggest that the inhibitory effect of antibody S121 was specific for HIV-1 on lymphoid cells.

Inhibition of virus entry

Since there was an inhibitory effect with certain antibodies directed against CD18, CD11b,c, and CD50 (CD50) the entire collection of antibodies directed against these antigens as well as those against CD54 were tested for their ability to inhibit the entry of VSV(HIV) pseudotype viruses bearing HIV-1 envelope glycoproteins. One antibody directed against CD18 and one directed against CD11c significantly inhibited the plating of VSV(HIV) pseudotype viruses (by 68% and 70% respectively) (Table 2). The antibody directed against CD11c that caused a 70% reduction in VSV(HIV) plating was the same antibody that significantly inhibited syncytium induction. None of the antibodies directed against CD11a inhibited syncytium formation and none of the antibodies directed against CD18 or CD11b inhibited the entry of VSV(HIV) pseudotype viruses (Table 2).

Table 2. *Inhibition assays using MAbs directed against adhesion molecules*

Antigen	No. of MAbs tested	No. that inhibited VSV(HIV) entry	No. that inhibited syncytium formation	MAbs that inhibited syncytium formation*					
						Ascites dilution:			
				Code	Name	1:50	1:100	1:500	1:5000
CD18	10	0	1	S123	6.7	+/−	+/−	++	++
CD11a	15	1 (68%)†	0						
CD11b	10	0	1	S172	CC1.7	+/−	+/−	++	++
CD11c	11	1 (70%)	1	S143	BU-15	+/−	+/−	+	++
CD50	17	8	1	S121	ICO-60	+/−	+/−	+	++
Controls									
CD4‡	1	90%	1	None	Q4120	+/−	+/−	+/−	+
No MAb		0	0			++	++	++	++

* +/−, Significant inhibition (80–90% reduction); +, partial inhibition (50% reduction); ++, syncytium formation, no reduction by antibody.

† Antibody S167, named G-25·2.

‡ Control antibody Q4120 was a purified antibody used at corresponding concentrations of 40 μg, 20 μg, 4 μg and 0·4 μg/ml.

*Inhibition assays of syncytium formation, VSV(HIV-1) pseudotype plating and infectivity using antibodies against ICAM-3 on SupT1 cells**

MAb code no.	MAb name	Domain	Syncytium formation with HIV-1*	Inhibition of VSV(HIV-1) plating (%)†	Inhibition of HIV-1 infectivity (%)
S081	BRIC79	1	++	0	0
S084	CG106	1	++	20	30
S087	CBRIC3/1	1	++	0	0
S088	CBRIC3/2	2	++	0	0
S089	CBRIC3/3	4	++	0	30
S090	CBRIC3/4	4	++	0	0
S091	CBRIC3/5	4	++	0	0
S092	CBRIC3/6	1	++	0	0
S093	WDS3A9	1	++	70±5·6	0
S106	BY44	1	++	0	0
S108	TP1/24	2	++	70±5·6	0
S109	HP2/19	1	++	75±4·9	60
S112	KS128	1 and 2	++	70±12·0	0
S113	152-2D11	1 and 2	++	60±9·1	70
S114	140-11	1	++	75±4·9	70
S115	101-1D2	2	++	70±4·2	0
S121	ICO-60	1	+/−	90±4·2	90
	Q4120		+/−	90±2·1	90
			++	0	0

* ++. No inhibition of syncytium formation. +/−. Significant inhibition of syncytium formation (80–90% reduction).

† Standard deviations are provided as a measure of variability for antibodies that significantly inhibited pseudotype plating.

Table 4. *Effect of antibodies S121 (anti-ICAM-3) and control Q4120 (anti-CD4) on VSV(HIV-1) pseudotype plating and HIV-1 infectivity*

	VSV(HIV-1) pseudotype plating P.f.u. counted at ascites dilution:*			HIV-1 infectivity p24 (pg/ml) in culture medium at ascites dilution:		
Antibody	1:50	1:100	1:500	1:50	1:500	1:5000
S121	2	11	54	0	0	130
Q4120	13	20	26	0	0	2900
No MAb	> 100	> 100	> 100	2000	2000	2300

* Approximately 100 p.f.u. of VSV(HIV-1) plated.

Of 17 antibodies directed against ICAM-3, only one (S121) significantly inhibited syncytium formation. This same antibody inhibited the plating of VSV(HIV) pseudotype viruses by up to 90%, a degree comparable to that of the control antibody directed against CD4 (Tables 3 and 4). Although the control anti-CD4 antibody did not neutralize VSV(HIV) plating totally, its inhibitory effect on pseudotype plating could be titrated further than for antibody S121 (Table 4). Seven other antibodies to ICAM-3 significantly inhibited VSV(HIV) pseudotype plating by up to 70% without having an effect on syncytium formation (Table 3); total inhibition was not achieved using these antibodies, not even at ascites dilutions of 1:25.

The observed inhibition of VSV(HIV) plating was not restricted to SupT1 cells, since equivalent results were also observed using the U937 and CEM cell lines.

Effect of anti-ICAM-3 antibodies on HIV infectivity

The antibodies directed against ICAM-3 were tested to determine their effect on the infectivity of HIV_{Lai} in culture. The antibodies that did not inhibit VSV(HIV) plating similarly had no effect on HIV infectivity on SupT1 cells. For antibodies S109, S113, S114 and S121, the inhibition of HIV_{Lai} infectivity was very similar to that of VSV(HIV) pseudotype plating (Table 3). Table 4 shows that antibody S121 inhibited HIV infectivity more effectively than the control anti-CD4 antibody at an ascites dilution of 1:5000. The difference between the efficiency of antibody S121 in the pseudotype assay and the infectivity assay at a 1:500 ascites dilution could reflect the higher number of cells used/volume of antibody in the pseudotype assay compared to the infectivity assay.

A discordant pattern was observed for antibodies S093, S108, S112 and S115 (Table 3). Although they inhibited VSV(HIV) plating by up to 70% they had a marginal effect on HIV_{Lai} infectivity. The pseudotype assay measures specifically entry in a single round of infection whereas the infectivity assay monitors several round of replication and virus spread in culture over

time. The avidity of antibody binding may therefore have been less stable for those antibodies showing a discordant pattern in the infectivity assay. In addition, Table 3 shows that antibodies S108 and S115 bind domain 2, S112 binds domains 1 and 2 whereas S093 binds domain 1. In contrast, the majority of antibodies that were inhibitory to both pseudotype plating and infectivity mapped to domain 1 on ICAM-3. Only four antibodies to domain 1 had no effect on HIV entry or infectivity. The precise locations and amino acid sequences of the epitopes recognized by the 17 anti-ICAM-3 antibodies is not presently known.

Discussion

HIV entry and HIV-induced syncytium formation are proving to be complex processes that are poorly understood in precise molecular terms. In an effort to identify candidate HIV-1 co-receptor molecules a panel of 166 MAbs raised against diverse adhesion molecules was initially screened for the ability of the MAbs to inhibit HIV-1-induced cell fusion. A large number of antibodies had no effect on HIV-1-induced cell fusion (Table 1); however, this does not exclude these molecules as candidate co-receptors, because insufficient antibodies to each antigen were tested to cover all known epitopes. Indeed, not all epitopes on human CD4 are involved in HIV-induced cell fusion, and it was in this way that the epitope corresponding to the HIV binding site on CD4 was identified (Sattentau *et al.*, 1986).

This study involved the testing of more antibodies directed against LFA-1 (CD18/CD11a) and CD11b, CD11c than previously described. In agreement with previous results (Golding *et al.*, 1992) a limited number of antibodies had a significant inhibitory effect on cell-to-cell fusion without affecting virus entry. Only one antibody raised against CD11a inhibited virus entry alone (Table 2). Previous studies have shown these molecules to be more relevant in the process of cell-to-cell fusion (syncytium formation) rather than in the fusion of viral and cellular membranes during virus entry (Goudsmith & Smit, 1990; Panteleo *et al.*, 1991).

The processes of cell-to-cell fusion and fusion between viral and cellular membranes may involve the participation of distinct components in addition to CD4. This hypothesis is supported by the fact that cell-to-cell fusion does not always reflect virus–cell fusion (infection) since some cell lines, e.g. RC2-A (Valentin *et al.*, 1994) and a subline of U937 (Collman *et al.*, 1989) will not undergo cell-to-cell fusion despite being susceptible to HIV infection. Furthermore, certain epitopes on CD4 have been reported to be involved in HIV-induced syncytium formation but not entry (Corbeau *et al.*, 1993) showing that these processes are also not identical regarding their involvement of CD4. The [illegible] is a highly qualitative assay, since different [illegible] lines vary in the number and extent of syncytia in response to HIV. We have now identified [illegible] that can distinguish between the two assays, for [illegible] by inhibiting cell-to-cell fusion but not virus [illegible] vice versa, emphasizing that these two processes, though involving membrane fusion, are not identical.

The results of this study provide the first [illegible] suggesting an involvement of ICAM-3 in HIV entry. [illegible] antibody to ICAM-3 (S121) significantly inhibited [illegible] only syncytium induction, but also the [illegible] VSV(HIV) pseudotype viruses and the infectivity [illegible] HIV_{Lai} (Tables 2, 3 and 4). The effect of S121 [illegible] syncytium induction was specific for HIV-1 on [illegible] cells since HIV-1-induced cell fusion of HeLa-CD4 [illegible] was not affected and nor was syncytium formation induced by HIV-2_{rod} on SupT1 cells (data not shown). Interestingly, an additional seven MAbs raised against ICAM-3 significantly inhibited the entry of VSV(HIV) pseudotype viruses without affecting syncytium formation. This inhibition of pseudotype plating was observed on CEM (T cell) and U937 (monocytoid) cells. The discordant effect noted for the four antibodies that significantly inhibited entry but not infectivity could reflect differences in the avidity of antibody binding to ICAM-3 and the stability of the antigen–antibody complex over time in culture. The majority of antibodies that showed this discrepancy bound domain 2 whereas those that were most effective bound domain 1 (Table 3). Since more antibodies were identified that affected virus entry rather than syncytium formation, this suggested that ICAM-3 is more relevant in the process of virus entry than in cell-to-cell fusion (syncytium formation).

The VSV pseudotype assay measured HIV-mediated entry independently of later steps in the HIV replicative cycle. Antibody was only present during VSV(HIV) adsorption and replication of VSV then proceeded in the absence of antibody. In contrast, the infectivity assay involved the continued presence of antibody in culture. No antibody inhibited HIV infectivity without inhibiting VSV(HIV) pseudotype plating, although the converse was true. This suggested that the observed inhibition did not result from indirect effects on cell viability or post-penetration HIV replication, but rather from a direct inhibition of virus–receptor interactions.

ICAM-3, a molecule of 120 kDa belonging to the immunoglobulin supergene family, was recently shown to be the same as CD50 (Juan *et al.*, 1993), and represents the third ligand for LFA-1 (De Fougerolles & Springer, 1992). It consists of five immunoglobulin-like domains having significant homology to ICAM-1, with the greatest divergence in the cytoplasmic tail (Fawcett *et al.*, 1992; Vazeux *et al.*, 1992; De Fougerolles *et al.*

ICAM-3 is expressed on lymphoid cells, dendritic and cells of the monocyte/macrophage lineage, ICAM-1, which is also found on epithelial cells. In way, ICAM-3 gene expression largely correlates with *in vivo* receptor host range of HIV.

The observations that HeLa–CD4 cells, which lack ICAM-3 gene expression, are nevertheless still susceptible to HIV-mediated cell fusion implies that HIV-1 the capacity to exploit different cell surface molecules different cell types in order to achieve membrane fusion and entry following binding to CD4 and that the co-receptors for HIV-1 on HeLa–CD4 and SupT1 cells are distinct. This is certainly plausible since HIV can utilize either CD4 (Dalgleish *et al.*, 1984; Maddon *et al.*, 1986) or galactosyl ceramide-mediated entry (Bhat *et al.*, 1991; Harouse *et al.*, 1991; Fantini *et al.*, 1993) depending on host cell type.

Constitutive expression of ICAM-3 on lymphoid cells makes it readily available to interact with HIV-1 on the cell surface in combination with CD4. Unlike ICAM-1, ICAM-3 is not only involved in LFA-1-mediated adhesion but also acts as a signal transducer providing potent co-stimulatory signals that lead to T cell activation following LFA-1 binding (Hernandez-Caselles *et al.*, 1993; Vives, 1994). It is conceivable that interactions between ICAM-3 and HIV-1 may similarly lead to cellular activation, a feature necessary for HIV replication since LFA-1 binds to the most extracellular domain of ICAM-3 (domain 1) in common with antibodies that significantly inhibited HIV-1-induced cell fusion and entry (Table 3; Klickstein *et al.*, 1993). ICAM-3 therefore represents an interesting candidate HIV-1 co-receptor worthy of further study to determine its exact role in HIV-1 entry.

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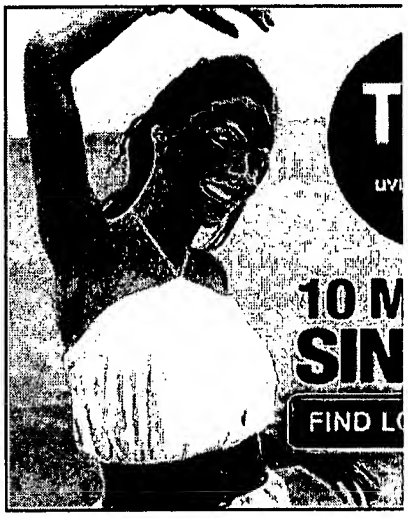

treatment

treatment

treat·ment [trtmnt] (*plural* treat·ments)

noun

Definition:

1. **provision of medical care:** the application of medical care to cure disease, heal injuries, or ease symptoms

2. **medical remedy:** a remedy, procedure, or technique for curing or alleviating a disease, injury, or condition
   - *a new treatment for asthma*

3. **way of handling somebody or something:** the particular way in which somebody or something is dealt with or

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bacteremia -->

bacteraemia

<microbiology> The presence of viable bacteria circulating in the bloodstream.

Origin: Gr. Bakterion, haima = blood

(11 Jan 1998)

Previous: backwoodsman, backworm, baclofen, bacon, Bacon's anoscope, bactenecin
Next: bacteremia, bacteri-, bacteria, bacteriaemia

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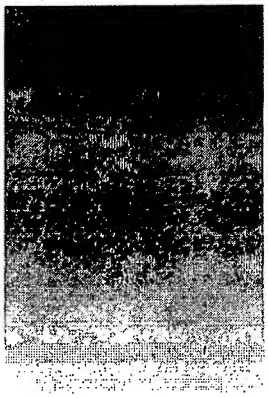

Vaccine-Preventable Diseases

Rubella

Rubella is a mild febrile viral disease, which mainly affects children; approximately one-half of rubella infections are subclinical. By far the most important clinical problem associated with rubella is the occurrence of congenital rubella syndrome (CRS) following infection of pregnant women. CRS can result in miscarriages, stillbirths, and fetal malformations, including congenital heart diseases, cataracts, deafness, and mental retardation. The risk of fetal damage is highest when maternal infection occurs just prior to conception or in the earliest months of pregnancy - 85% of CRS cases occur with infection in the first trimester - and is very rare after the twentieth week of pregnancy. Infected infants may appear normal at birth and fetal malformations may not become apparent for several years. Congenital infection can become chronic, and may result in diabetes and panencephalitis later in life. The costs associated with long-term care for cases of CRS represent a huge economic burden for affected families and for society at large ($514,000 per case on average).

Vaccination against rubella was introduced in Canada in 1969. Since the mid-1970s, rubella incidence in Canada has

remained relatively low (Figure 10). An average of approximately 1,000 cases (ranging from 237 to 2,450) were reported annually from 1986 to 1995; this represents a mean rate of 4.0 per 100,000 population. A number of college and university outbreaks have been reported in recent years. About one-third of the rubella cases reported in the last 5 years have been among adolescents 10 to 19 years of age. Overall, 50% to 60% of reported cases in Canada occur in persons between the ages of 10 and 39 years. Thirty-two cases of CRS were reported in Canada from 1986 to 1995; however, CRS is believed to be grossly underreported.

The primary objective of vaccination against rubella is to prevent infection during pregnancy. In addition to routine vaccination of children, vaccination is also recommended for all females of childbearing age unless they have documented prior immunization, or laboratory evidence of detectable antibodies from natural infection or previous immunization. Protection of pregnant women and women of childbearing age can be further ensured by vaccination of males, particularly those likely to come into contact with women at risk (such as males in secondary, post-secondary, and health-care institutions).

1998 Update: In 1998, rubella incidence fell to the lowest number ever recorded nationally at 0.2/100,000 or 67 cases (Figure 4). The majority of cases were diagnosed in the 15- to 19-year-old age group. The highest age-specific incidence was in the younger than one-year-old age group at 2.2/100,000 (eight cases) followed by the one- to four-year-old age group at 0.8/100,000 (13 cases). The male to female ratio was 1:2.

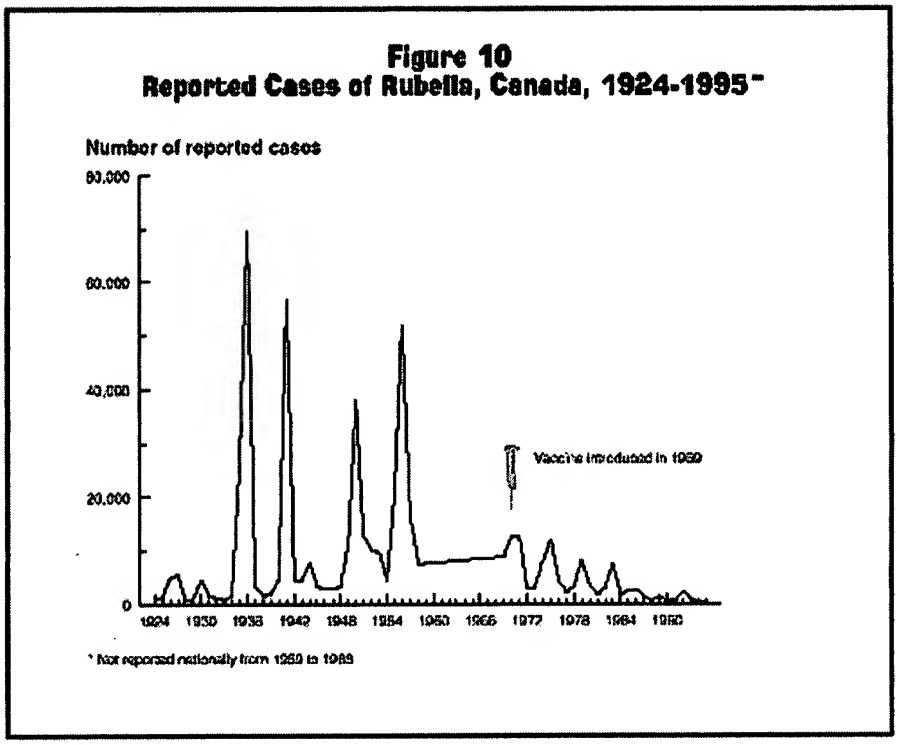

Figure 4) *Crude incidence of rubella reported in Canada, 1924 to 1998. No national reporting between 1959 and 1968*

Congenital rubella

Paediatrician-based active surveillance through the CPSP began in January 1996, and, since April 1996, cases reported to IMPACT have been forwarded to the CPSP. Paediatricians participating in CPSP are also asked to report infants with congenital rubella infection (CRI), defined as cases with no clinically compatible manifestations but with laboratory confirmation of infection. Given that the majority of infants infected with rubella in utero have no detectable clinical abnormalities at birth and many will go on to develop late-onset manifestations, it is important that CRI cases be identified.

Table 4 shows the number of CRS cases by year of reporting to surveillance systems in Canada from 1996 to 1998. Five older children previously diagnosed but not reported to NDRS were captured by active surveillance in 1996 and 1997. Of the newly diagnosed cases from 1996 to 1998, three were newborns and one was an adolescent with

late-onset manifestations.

TABLE 4: Congenital rubella syndrome (CRS) by year of reporting to Canadian Paediatric Surveillance Program (CPSP)/the Immunization Monitoring Program ACTive (IMPACT) Network and Notifiable Diseases Registry System (NDRS), January 1996 to December 1998

	Number of CRS reported to CPSP/IMPACT			Total number of CRS reported to NDRS
Year of reporting	Newly diagnosed	Previously diagnosed but not reported	Total	
1996	1	3	4	2
1997	2	2	4	1
1998	1	0	1	1

Table 5 shows the number of CRS cases by year of birth reported in Canada from 1996 to 1998. Since 1996, with enhanced surveillance through CPSP in place, only one to two reports of children born with CRS per year (0.3 to 0.5/100,000 births) have occurred.

TABLE 5: Congenital rubella syndrome by year of birth reported to Canadian Paediatric Surveillance Program (CPSP)/the Immunization Monitoring Program ACTive (IMPACT) Network and Notifiable Diseases Registry System (NDRS), January 1996 to December 1998

	Reported to			
Year of birth	NDRS only	CPSP/IMPACT only	NDRS and CPSP/IMPACT	Total
1996	1	0	1	2
1997	0	0	1	1
1998	0	0	1	1

Table 6 shows the CPSP data on the characteristics of the three cases born in Canada from 1996 to 1998. Two cases were infants of foreign-born mothers from countries where rubella immunization was not routine. Two cases were potentially preventable because the mothers were tested to be rubella-susceptible during a previous pregnancy but did not receive vaccination.

TABLE 6: Characteristics of infants with congenital rubella syndrome born in Canada from 1996 to 1998

Year of birth	Laboratory-confirmed	Clinical manifestations	Mother born in Canada	Previous maternal rubella IgG screening test	Maternal rubella immunization
1996	Yes	Multiple	No	Positive	Unknown
1997	Yes	Multiple	No	Negative	No
1998	Yes	Multiple	Yes	Negative	No

Ig Immunoglobulin

So far, no CRI has been reported to CPSP. The degree of underdiagnosis and under-reporting for CRI, CRS with less severe manifestations and CRS with late-onset manifestations is unknown.

Between July 1, 1996 and June 30, 1998, 145 women aged 15 to 44 years with positive RIgM were reported by five (24%) of 21 laboratories participating in the Rubella-Associated Adverse Pregnancy Outcomes Surveillance System (RAAPO). Twenty-one (14.5%) of the eligible cases were pregnant at the time of RIgM testing, of which 19 were from Manitoba where a large outbreak of rubella occurred. Of 14 pregnant women with known immunization histories, eight (57%) had been immunized against rubella, of whom four had been vaccinated less than nine months

before IgM testing. Of the 17 pregnant women who did not receive recent rubella vaccination, 11 (64%) had clinically 'healthy' newborns, and six (36%) had adverse pregnancy outcomes, including four induced abortions, one spontaneous abortion and one fetal death. RAAPO failed to capture three CRS cases identified by the other surveillance systems during the same surveillance period. Further evaluation of RAAPO, especially an assessment of under-reporting, is needed.

In summary, rubella infection in women of childbearing age continues to occur in Canada, but the number of newborns with CRS appears to be on a decline. There have been no reports of children with CRI to the CPSP or RAAPO. Based on the data from RAAPO, 36% of pregnant women with serologically confirmed rubella infection experienced fetal loss.

Rubella Vaccine

The rubella virus vaccine currently licensed in Canada incorporates live attenuated virus strain RA 27/3, prepared in human diploid cell culture. It is available as a monovalent vaccine or in combination with mumps and measles vaccines (MMR) or measles vaccine (MR). The vaccine is lyophilized and should be reconstituted just before administration with the diluent provided.

Efficacy and Immunogenicity

Rubella vaccine stimulates the formation of antibody to rubella virus in over 97% of susceptible individuals. Titres are generally lower than those observed in response to natural rubella infection.

Asymptomatic re-infection, manifest by a rise in antibody, has been observed in vaccinees and may account for the continued endemicity of rubella. Asymptomatic re-infection has also been observed in women with naturally acquired immunity and very low antibody titres. Rarely, transient viremia can occur in people immune by either natural disease

or prior immunization, but transmission to the fetus in this circumstance is believed to be rare.

Schedule and Dosage

Immunization schedules and requirements for MMR vaccine vary by province/territory and can be obtained from the local public health department. The dose of rubella vaccine, given either alone or combined with measles vaccine or measles and mumps vaccines, is 0.5 mL given as subcutaneous injection.

Infants and children

One dose of live rubella vaccine is recommended routinely for all children on or as soon as practical after their first birthday in combination with measles and mumps vaccines. Rubella vaccine should not be administered prior to 12 months of age. In all provinces and territories, a second dose of rubella vaccine is given at the time of the second dose of measles vaccine, administered at 18 months of age or at school entry and at least 1 month after the first dose. Although a second dose of rubella vaccine is not believed to be necessary for achieving elimination of CRS, it is not harmful and may benefit those who do not respond to primary immunization (1% to 3% of people).

Adolescents and adults

Rubella vaccine should be given to all female adolescents and women of childbearing age unless they have proof of immunity, which is either a record of prior immunization or laboratory evidence of detectable antibody. At the first visit, rubella immunization status should be assessed. If there is no documentation of prior immunization, one dose of rubella vaccine should be given, preferably as MMR vaccine, since a high proportion of women susceptible to rubella are likely also susceptible to measles. A clinical history of rubella without laboratory confirmation is not a reliable indicator of immunity.

Every effort should be made to immunize foreign-born adolescents and women from countries where rubella vaccine is in limited use (see Epidemiology section in the Guide) as soon as possible after entry to Canada or, for women who are pregnant upon presentation, immediately post-partum.

Since up to one-third of cases of CRS occur in second and subsequent pregnancies, it is essential that all women found to be susceptible during pregnancy receive rubella vaccine (preferably given as MR or MMR vaccine) in the immediate postpartum period and as soon as practical after delivery. Every effort should be made to immunize before hospital discharge. Canadian, U.S. and U.K. studies show that a large proportion of rubella-susceptible women are not immunized post-partum. Hospital standing order policies have been shown to be effective in increasing post-partum immunization rates.

In educational institutions, such as schools, colleges and universities, particular emphasis should be placed on immunization of susceptible female staff and female students of childbearing age because of their relatively high risk of exposure. In health care settings, the rubella immune status of female employees of childbearing age should be carefully reviewed, and those without documented immunity should be immunized. In addition, vaccine should be given to susceptible people of either sex who may expose pregnant women to rubella.

Booster Doses and Re-immunization

Antibody levels developed in response to earlier rubella vaccines decline over time, but this decline may not have great significance since any detectable antibody generally protects against viremic infection. The duration of protection is not yet known, but studies indicate that the duration of both cellular and humoral immunity exceeds 20 years. Booster doses are not considered necessary but are not harmful and may provide a marginal protective benefit in the population.

Serologic Testing

Pre-immunization: A documented history of immunization is presumptive evidence of immunity. Serologic screening in a person without documented immunization is neither necessary nor recommended, and may result in a missed opportunity to immunize.

Post-immunization: Serologic testing after immunization is unnecessary. Women of childbearing age without a prior record of immunization who are tested and found to be non-immune serologically should be offered one dose of rubella-containing vaccine. Those with a prior record of immunization who are serologically non-immune may be offered immunization, but such tests are likely to be falsely negative. It is not necessary to repeat immunization even if subsequent serologic tests are also negative, because such individuals usually have other evidence of rubella immunity. Prenatally: Serologic testing for rubella antibody should be a routine procedure during prenatal care for those without written serologic evidence of immunity or prior immunization. Prenatal testing in Ontario and Quebec indicates rates of serosusceptibility of about 7% and 7% to 11% respectively.

Storage Requirements

Rubella-containing vaccines should be stored in the refrigerator at a temperature of 2o C to 8o C. Once reconstituted, the vaccine should be administered promptly.

Simultaneous Administration with Other Vaccines

Rubella-containing vaccines may be administered at the same time but at a separate injection site as DPT-containing vaccines routinely given at 18 months and school entry, as well as adult tetanus-diphtheria vaccine. When administered at the same time as live virus vaccines other than measles and mumps, rubella-containing vaccine(s) should be given at a separate injection site or, if possible,

separated by a 4-week interval.

Adverse Reactions

Rash and lymphadenopathy occur occasionally. Acute transient arthritis or arthralgia may occur 1 to 3 weeks after immunization, usually persists for 1 to 3 weeks, and rarely recurs. These reactions are uncommon in children, but the frequency and severity increase with age, and they are more common in post-pubertal females, among whom arthralgia develops in 25% and arthritis-like signs and symptoms in 10% after immunization with RA 27/3. Recently published studies indicate no evidence of increased risk of new onset chronic arthropathies or neurologic conditions in women receiving RA 27/3 rubella vaccine. Paresthesia or pain in the extremities lasting 1 week to 3 months has been reported rarely. However, both the frequency and severity of adverse reactions are less than those associated with natural disease. Serious adverse reactions are rare. There is a growing body of literature to suggest a genetic predisposition to joint manifestations following rubella immunization. However, these manifestations are more serious after natural infection, and immunization against rubella among such people is indicated.

Contraindications

Administration of live rubella vaccine during pregnancy should be avoided because of the theoretical risk of CRS in the fetus.

Rubella vaccine should not be administered to people known to be hypersensitive to the vaccine components, such as antibiotics, used in its preparation; such reactions include anaphylactic hypersensitivity to neomycin. Convincing evidence supports the safety of routine administration of MMR vaccines to all children who have allergy to eggs. Fewer than 2 per 1,000 immunized egg-allergic children have been found to be at risk of anaphylactic reaction to MMR vaccine (see the chapter on Measles Vaccine in the

Guide for further details).

Precautions

Women of childbearing age should be advised to avoid pregnancy for 1 month after immunization. This recommendation is based on the duration of viremia after natural infection and evidence of vaccine safety.

Rubella vaccine is occasionally administered to women who were unknowingly pregnant at the time or who became pregnant shortly after immunization. Reassurance can be given that no fetal damage has been observed in the babies of over 700 susceptible women who received vaccine during their pregnancy and carried to term. The theoretical risk of teratogenicity, if any, is very small. Therefore, receipt of rubella vaccine in pregnancy, or conception within 1 month after receipt, should not be a reason to consider termination of pregnancy.

Breast-feeding is not a contraindication to rubella immunization. Although vaccine virus has been detected in breast milk and transmission can occur, no illness has been reported in the infants.

As with other live vaccines, rubella vaccine should not be administered to people whose immune mechanism is impaired as a result of disease or therapy, except under special circumstances (see section on Immunization in Immunocompromised Hosts in the Guide). These vaccines would generally be administered to provide protection against measles. The immune response in such individuals may be impaired. Rubella-containing vaccines may be administered to HIV-infected people who are not severely immunosuppressed and among whom use of the vaccine has not been associated with serious adverse reactions.

Other Considerations

Small quantities of vaccine strain virus may be detected in the nasopharynx of some vaccinees 7 to 28 days after

immunization, but the risk of transmission to contacts seems to be very low. After many years of vaccine use, only a few cases of possible transmission have been documented; in only one instance was the contact known to be previously immune by serologic testing. Therefore, it is safe to administer vaccine to those who are in contact with susceptible, pregnant women and with immunocompromised people.

Anti-Rho(D) immune globulin may interfere with response to rubella vaccine. Rubella-susceptible women who receive anti-Rho(D) immune globulin post-partum should either be given rubella vaccine at the same time and tested 3 months later for rubella immunity, or should be immunized with rubella vaccine 3 months postpartum, with follow-up ensured.

Vaccine must not be administered less than 2 weeks before an immune globulin injection. When immune globulin has been administered, rubella immunization should be delayed for 3 months; it should be delayed for 5 months if given as MMR vaccine (see Chapter on Passive Immunizing Agents in the Guide.) It has been shown that previous or simultaneous blood transfusion does not generally interfere with the antibody response to rubella immunization. In such cases, however, it is recommended that a serologic test be done 6 to 8 weeks after immunization to test the individual's immune status. If the individual is seronegative, a second dose of vaccine should be administered.

Passive immunization

The effectiveness of immune globulin for post-exposure prophylaxis of rubella is unknown and as such is not recommended.

Management of outbreaks

During outbreaks, people at risk who have not been immunized or do not have serologic proof of immunity should be given vaccine promptly without prior serologic testing. A history of rubella illness is not a reliable

indicator of immunity. Even though rubella immunization has not been shown to be protective when given after exposure, it is not harmful. It will protect the individual in future if the current exposure does not result in infection.

Surveillance

All suspected and confirmed cases of rubella and CRS must be reported to the appropriate local or provincial/territorial public health authority. In addition to this passive surveillance, CRS is monitored through the Canadian Paediatric Surveillance Program.

Laboratory confirmation is carried out by serodiagnostic laboratory methods or culture. The specific diagnosis is particularly important in suspect cases who are contacts of pregnant women and in suspect cases of CRS, as well as during outbreaks. A significant, rising antibody titre from acute and convalescent serum samples is confirmatory, the first sample being taken within the first 7 days after illness and the second 10 days after the first. Rapid confirmation may be obtained by testing for rubella-specific IgM antibody in a serum sample taken between 3 days and 1 month after rash onset. There may be false-negative results if the serum sample is taken too early or too late after the clinical illness, and false positives occur frequently, since the test has low positive predictive value outside the outbreak setting. Congenital infection may be confirmed by isolation of the virus in neonatal urine or nasopharyngeal secretions, detection of IgM antibody to rubella virus in blood, or the persistence of antibody to rubella virus beyond the age of 3 months. Consultation with the regional public health laboratory will indicate the availability and applicability of various diagnostic methods for rubella.

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HIV VACCINES

Back to primary school

John P. Moore

ATTACK by the human immunodeficiency virus on T cells involves binding of the viral envelope gp (for glycoprotein) 120 subunit to the cells' CD4 receptor. Binding is followed by fusion of virus and cell, the first step in the viral replication cycle. Because gp120 is central to this process, using gp120 subunit vaccines to generate neutralizing antibodies against HIV-1 has been at the forefront of vaccine strategies for many years. Yet there has been vigorous debate about whether such an approach will work.

That debate now takes on a new dimension with the appearance of a paper by Sullivan *et al.* in *Journal of Virology*[1]. In general, the findings bear on the issue of the relative resistance of primary strains of HIV-1, as opposed to strains highly passaged *in vitro*, to neutralizing antibodies, and whether such variable resistance is real or an assay-dependent artefact. In particular, the new results may help explain why antibodies raised to current monomeric gp120 vaccines do not neutralize primary viruses[2,3].

Neutralization

Sullivan *et al.* have measured the interactions of monoclonal antibodies (mAbs) with the native, oligomeric forms of the HIV-1 envelope glycoproteins, found on virions and virus-infected cells, and compared the results with levels of virus neutralization. They draw various conclusions. First, they confirm that mAb interactions with monomeric gp120 do not predict the extent of virus neutralization[4]. Second, they take things further and show that mAb reactivity with oligomeric glycoprotein is a good correlate of neutralization, which is consistent with observations on HIV-1 LAI from Sattentau and myself[5].

The third conclusion is that mAbs don't bind nearly as well to oligomers from primary viruses as to oligomers from strains whose envelope glycoproteins have been altered by passage in T-cell lines *in vitro*[1,6,7]. The relative inaccessibility of neutralization sites on primary viruses, including the V3 loop, combined with Sullivan and colleagues' observation that such viruses have a greater density of surface glycoproteins, may account for much of the mechanism behind neutralization resistance. HIV-1 has presumably evolved these defences to counter the abundant antibodies raised against it in infected humans, perhaps at the expense of some loss of replication efficiency[1,4]. As disease progresses and the immune system collapses, the more virulent strains that can emerge may need less protection.

Resistance of primary virus to neutralization was first documented from studies on soluble CD4 (sCD4)[8], and is known to be a function of the oligomeric structure of the envelope glycoproteins[9]. But it has not been widely appreciated that resistance to neutralization by antibodies probably has the same basis, in that sCD4 resistance is an indirect consequence of a more natural phenomenon. Soluble CD4 strongly neutralizes strains of HIV-1 adapted to T-cell lines; yet it increases the infectivity of certain strains of simian immunodeficiency virus and HIV-2[10], which led to the proposal that SIV's capacity for fusing with a cell ('fusogenicity') is activated by binding of CD4[11].

It now seems clear that HIV shares this property. Sullivan *et al.* show that sCD4 has a biphasic effect on the fusogenicity of two strains of primary HIV-1, increasing it at low concentrations, but inhibiting it at higher concentrations as gp120–sCD4 complexes are stripped from the virus. Schutten *et al.*[12] have also observed sCD4-enhanced fusogenicity of primary HIV-1 clones. Previous studies with sCD4 helped create the perception that the envelope glycoproteins of HIV-1 were in some critical way different to those of other primate lentiviruses. But it now seems clear that the anomalous viruses are the highly adapted strains of HIV-1 such as LAI and MN, not HIV-1 *per se* — a lesson we are learning, once again, the hard way.

Yet perhaps even more striking are results stemming from Sullivan and colleagues' experiments with a human mAb (F105) to a complex gp120 epitope near the CD4-receptor binding site. F105, they find, not only fails to neutralize a primary strain of HIV-1, but actually increases its infectivity. It does, however, effectively neutralize strains that are adapted to T-cell lines.

These observations are akin to those with sCD4 — by binding to an epitope overlapping the CD4-binding site, an antibody seems to be able to trigger some of the fusion-activating conformational rearrangements in the viral envelope. As different mAbs to this epitope cluster induce different conformational changes in gp120 monomers, whether an antibody mediates activation of fusion[1,12], or neutralization[1,4], might be a subtle function of the geometry of its epitope in relation to the CD4-binding site. Schutten *et al.*[12] have also shown that two other human mAbs to CD4-binding-site epitopes, and one to the V3 loop, increase the fusogenicity of a primary HIV-1 clone; the latter finding is in itself notable, for there is a growing understanding that the V3 loop is not the principal neutralizing determinant for primary viruses.

What are the implications of all this for vaccine development? Several high-quality, monomeric gp120 vaccines have been tested in humans. One advantage of the correctly folded forms of these proteins has been their ability to induce antibodies to the complex epitopes surrounding the CD4 binding site; indeed rodent mAbs have been raised against gp120 that are indistinguishable from antibodies made in infected humans[13]. Yet although sera from gp120-immunized animals and humans neutralize viruses adapted to T-cell lines, they are inactive against primary strains of HIV-1[2,3].

Infectivity

There is, as yet, no evidence that the increase in infectivity mediated by F105-like antibodies contributes to such lack of activity, but the possibility should now be considered. We have found that many sera from HIV-1 infected humans, selected for high titres of anti-gp120 antibodies, weakly increase the infectivity of primary HIV-1 strains; other sera merely lack net neutralizing activity (Y. Cao *et al.*, unpublished data). Perhaps F105-like antibodies contribute to this effect by counteracting, wholly or in part, any neutralizing antibodies present (although the involvement of antibodies that increase infectivity in more conventional ways[14] should not be discounted).

The present generation of monomeric gp120 immunogens seems unable to breach HIV-1's defences, and Sullivan *et al.* show just how formidable is the wall that lies across the path to an antibody-mediated, anti-HIV vaccine. We now must decide whether to blast a way through this barrier, subtly undermine it or even choose another route entirely. But brute force is rarely the answer, so the need for a large clinical infrastructure to evaluate the available gp120 vaccines should be reconsidered. □

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